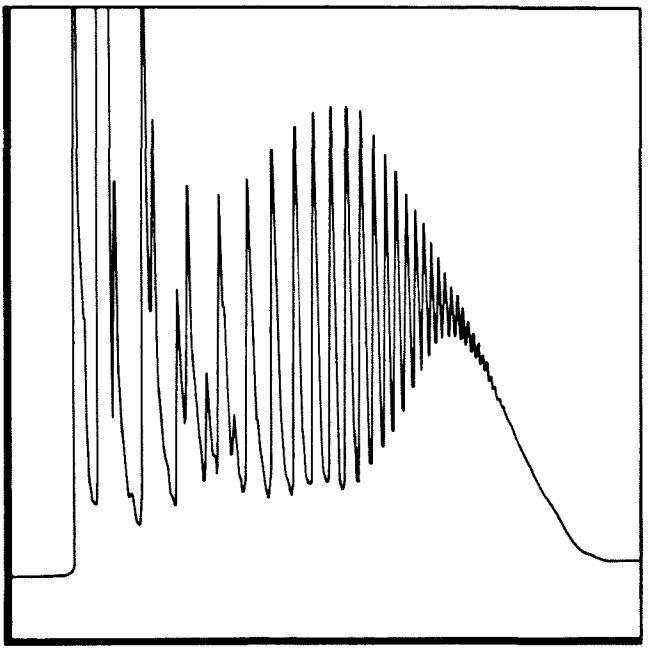
Oligomerization Reactions of Nucleotide Analogs



J. Visscher

Oligomerization Reactions of Nucleotide Analogs

(Oligomerisatie reacties van nucleotide analogen)

Oligomerization Reactions of Nucleotide Analogs

een wetenschappelijke proeve op het gebied van
de natuurwetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Katholieke Universiteit te Nijmegen,
volgens besluit van het college van decanen
in het openbaar te verdedigen op
maandag 8 januari 1990 des namiddags te 3.30 uur

door

JOHANNES VISSCHER
geboren op 13 oktober 1956
te Appingedam

Offsetdrukkerij van de β-faculteiten
Nijmegen

Promotor : Prof. dr. A.W. Schwartz

The investigations reported in this dissertation were partially supported by U.S. National Aeronautics and Space Administration grant NGR 05067.

Aan allen die ik lief heb

Al degenen, die op enigerlei wijze hebben bijgedragen aan het tot stand komen van mijn proefschrift, wil ik hartelijk bedanken.
Mijn promotor, prof. dr. Alan W. Schwartz, ben ik zeer erkentelijk voor de wijze waarop hij het onderzoek heeft begeleid. Prof. dr. G.I. Tesser bedank ik voor het zorgvuldig becommentariëren van dit manuscript. Mijn (ex)collega's Cor Bakker, René Tros en Rob van der Woerd bedank ik voor het prettig samenwerken. De medewerkers van de tekenkamer, fotografie en offsetdrukkerij wil ik bedanken voor hun hulp bij het drukken van dit proefschrift. Tenslotte, maar in het bijzondere, ben ik veel dank verschuldigd aan mijn ouders en Carla.

This dissertation is based on the following articles:

Chapter 2 "Nucleic acid-like structures II. Polynucleotide analogues as possible primitive precursors of nucleic acids", A.W. Schwartz, J. Visscher, C.G. Bakker and J. Niessen. Origins of Life 17, 351-357 (1987).

Chapter 3 "Nucleic acid-like structures III. Oligomerization of 3'-deoxyadenosine-2',5'-diphosphate", J. Visscher and A.W. Schwartz. J. Mol. Evol. 26, 291-293 (1987).

Chapter 4 "Oligomerization of deoxynucleoside-bisphosphate dimers", J. Visscher R. van de Woerd, C.G. Bakker and A.W. Schwartz. Origins of Life 19, 3-6 (1989).

Chapter 5 "Template-directed synthesis of acyclic oligonucleotide analogues", J. Visscher and A.W. Schwartz. J. Mol. Evol. 28, 3-6 (1988).

Chapter 6 "Manganese-catalyzed oligomerizations of nucleotide analogues", J. Visscher and A.W. Schwartz. J. Mol. Evol., in press.

Chapter 7 "Oligomerization of cytosine-containing nucleotide analogs in aqueous solution", J. Visscher and A.W. Schwartz. J. Mol. Evol., in press.

Chapter 8 "Template-directed oligomerization catalyzed by a polynucleotide analog", J. Visscher, C.G. Bakker, R. van der Woerd and A.W. Schwartz. Science 244, 329-331 (1989).

Contents

Chapter 1

Inleiding

1.1 De geschiedenis van de RNA-wereld

Door microfossielen, die in 3.5 miljard jaar oud gesteente zijn gevonden, is aangetoond dat 1 miljard jaar na de vorming van de aarde al verschillende levensvormen bestonden. (Miller and Orgel, 1974). Daar geen gegevens bekend zijn betreffende de chemische evolutie gedurende de eerste miljard jaar, is het proces tot vorming van dit leven onbekend. Algemeen wordt aangenomen dat de ontwikkeling van zelfreplicerende systemen vanuit prebiotische materialen de drijvende kracht achter de chemische evolutie was.

Er zijn verschillende hypotheses gedefinieerd met betrekking tot de oorsprong van het leven (Dose, 1987; Miller and Orgel, 1974). Een van de meest besproken hypotheses is dat de chemische evolutie is gebaseerd op de replicerende eigenschappen van nucleïnezuren (Crick, 1968; Orgel, 1968). Deze hypothese wordt ondersteund door het theoretische werk van Eigen en Schuster (1982) betreffende de zelfreplicatie van polynucleotiden, en het experimentele werk verricht door Orgel en collega's op het gebied van niet enzymatische replicatie-reacties (Orgel, 1986).

Door de ontdekking van ribozymen kreeg deze hypothese een grote belangstelling (Cech , 1986 en 1989; Darnell and Doolittle, 1986; Lewin, 1986; Gilbert, 1986; North, 1987). Rybozymen, RNA molekulen die sequentie specifieke transesterificaties kunnen uitvoeren, combineren informatieve en functionele eigenschappen in een macromolecuul (Cech, 1987; Guerrier-Takada et al., 1983; Zaug and Cech, 1986).

De L-19 intervening sequence van de self-splicing r-RNA precursor van *Tetrahymena thermophila* katalyseert nucleotide transesterificatie, waarbij polycytidyl zuren uit pentacytidyl zuren gesynthetiseerd worden. (Zaug and Cech, 1986) Doudna and Szostak (1989) modificeerden dit ribozym waarbij de substraat bindende matrijs van het katalytisch centrum werd gescheiden. Dit gemodificeerde ribozym is in staat om op een willekeurige matrijs de condensatie van complementaire oligonucleotiden te katalyseren. Met de ontdekking van ribozymen werd de RNA-wereld theorie geintroduceerd. Volgens deze theorie is het huidig leven geëvolueerd vanuit een primitieve levensvorm, die gebaseerd is op zelf replicerende RNA moleculen. (Gilbert, 1986).

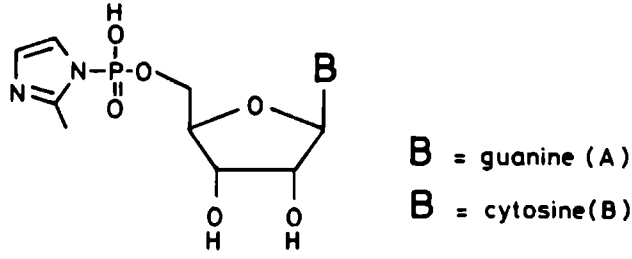
1.2 Matrijs-afhankelijke reacties

Met als doel het ontwikkelen van een model systeem voor spontane replicatie van RNA-moleculen, bestudeerden Orgel en medewerkers enzym-vrije oligomerisatie reacties in aanwezigheid van matrijzen (Orgel, 1986). Deze reacties zijn gebaseerd op de vorming van dubbele of drievoudige helicale complexen tussen polynucleotiden en hun complementaire mononucleotiden (Saenger, 1984). Door deze complexvorming worden de nucleotiden gericht in een voor de reactie noodzakelijke oriëntatie. De eerst vermelde matrijs afhankelijke reactie was de door poly(A) gekatalyseerde koppeling van twee hexanucleotiden $(pT)_6$ tot de dodecanucleotide $(pT)_{12}$ (Naylor and Gilham, 1966).

Korte oligonucleotiden van adenylzuur of guanylzuur kunnen gesynthetiseerd worden uit de geaktiveerde mononucleotiden en hun complementaire matrijzen (Sulton et al., 1968 en 1969; Lohrman and Orgel, 1978). Door de grotere nucleofilie van de 2'-OH groep van het cis-diol systeem van ribose worden er voornamelijk 2',5'-fosfodiëster bindingen gevormd. Met behulp van 2-waardige metaalionen kunnen deze reacties in effectiviteit en regioselectiviteit beinvloed worden. Het rendement van condensatie reacties van geactiveerd adenylzuur, gekatalyseerd door een poly(U) matrijs wordt sterk verbeterd door Pb^{2+} ionen, terwijl het gehalte aan 3',5'-fosfodiëster bindingen binnen de gevormde oligomeren toeneemt (Sleeper et al., 1979). Een dergelijk effect hebben ook Zn^{2+} ionen op de door poly(C) gekatalyseerde

oligomerisatie reactie van geactiveerd guanylzuur. Tijdens deze reactie ontstaan hoofdzakelijk oligomeren met 3',5'-fosfodiëster bindingen (Bridson and Orgel, 1980).

Een regiospecifieke 3',5'-fosfodiëster koppeling wordt gevonden bij de gekatalyseerde oligomerisatie reactie van $\underline{1}^{A}$ (Inoue and Orgel 1982). In tegenstelling tot de purine nucleotiden falen deze oligomerisatie reacties bij geactiveerde pyrimidine nucleotiden. Een poly(G) matrijs kan de oligomerisatie van cytidylzuur niet richten, omdat poly(G) zelf een stabiele viervoudige helix vormt. Een reactie van geactiveerd uridylzuur op een poly(A) matrijs is niet mogelijk doordat de onderlinge stacking met de uridine ringen te gering is om een stabiel poly(A):U complex te vormen (Saenger, 1984).



1

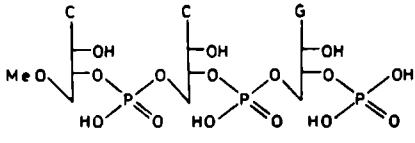
Figuur 1.1: De structuren van; $\underline{1}^{A}$ guanylzuur 2-methylimidazolide, $\underline{1}^{B}$ cytidylzuur 2-methylimidazolide.

Een poly(C,U) random copolymeer, dat rijk is aan cytidine, katalyseert de oligomerisatie van de complementaire geactiveerde mononucleotiden adenosine en guanosine (Inoue and Orgel, 1983; Joyce and Inoue et al., 1984). De inbouw van geactiveerde mononucleotiden in de gevormde oligomeren verloopt met adenylzuur trager dan met guanylzuur. Door verschuiving van de nieuw gevormde oligomeer op de copolymeer onstaan niet-complementaire G:U paren, waardoor de uridine plaatsen op de poly(C,U) copolymeer afgeschermd worden. Met de random copolymeren poly(C,G) (Joyce et al., 1986) en poly(C,A) (Joyce et al., 1988) zijn vergelijkbare resultaten verkregen.

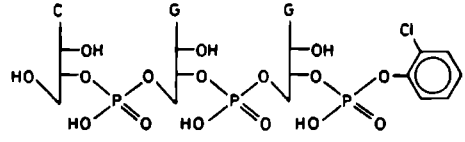
Dat een sequentie onder bepaalde omstandigheden in zijn complement kan wor-

den omgezet, wordt door Inoue et al. (1984) aangetoond. De door poly(CCGCC) gekatalyseerde oligomerisatie reactie van mengsels van $\underline{1}^A$ en $\underline{1}^B$, geeft verschillende cytidine en guanosine bevattende oligomeren. De gevormde tetra- en pentameren bestaan voornamelijk uit de complementaire sequenties GGCG en GGCGG. Andere sequentie specifieke oligomerisaties zijn beschreven door Heartly en Orgel (1986), en door Acevedo en Orgel (1987).

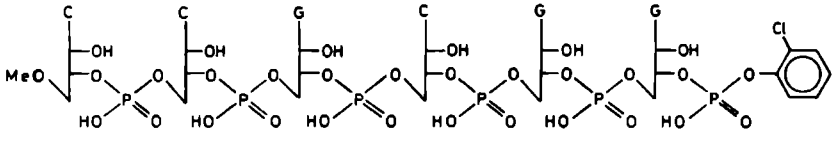
Uit het onderzoek van Orgel en medewerkers blijkt dat een effectieve oligomerisatie alleen mogelijk is wanneer de matrijs een overmaat aan cytosine resten bevat. Een zelf-reproducerend systeem zal dus onder deze omstandigheden niet kunnen ontstaan. Een speciaal geval van een zelf-reproducerend systeem is ontwikkeld door Kiedrowski (1986). De koppeling tussen de beschermde trimeren $\underline{2}$ en $\underline{3}$ wordt gekatalyseerd door het gevormde zelfcomplementaire produkt $\underline{4}$. Deze reactie heeft in de prebiotische evolutie echter geen betekenis omdat ze, door de gebruikte beschermgroepen, naar één produkt wordt gericht, waardoor geen mutatie of selectie uit een grote populatie mogelijk is.



$\underline{2}$



$\underline{3}$



$\underline{4}$

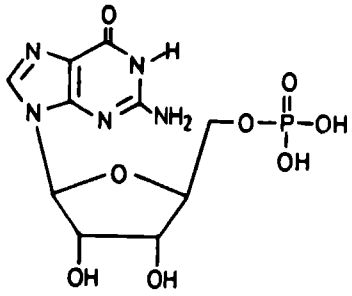
Figuur 1.2: De structuren van $\underline{2}$ de 5'-hydroxy beschermde trimeer d(5'-Me-CCGp-3'), $\underline{3}$ de 3'-fosfaat beschermde trimeer d(5'-CGGp-OPhCl-3'), en $\underline{4}$ de hexameer d(5'-Me-CCGCGGp-OPhCl-3').

1.3 Een replicatie systeem gebaseerd op nucleotide-analogen

Hoewel het idee, dat de eerste levensvorm was gebaseerd op zichzelf replicerende RNA-moleculen, een grote bekendheid heeft gekregen (Gilbert, 1986), is de ontwikkeling van zo'n systeem prebiotisch gezien alles behalve duidelijk. Een van de problemen is de beschikbaarheid van de mononucleotiden in een prebiotisch milieu. De vorming van suikers, via de formose reactie (een autokatalytische polymerisatie-reactie van formaldehyde), geeft naast het gewenste ribose, een complex mengsel van koolhydraten (Shapiro, 1988). Een koppeling van ribose met een purine verloopt zeer inefficiënt onder omstandigheden die geologisch gezien "realistisch" zijn (Fuller et al., 1972), terwijl de reactiviteit van een pyrimidine base ten opzichte van ribose vrijwel geheel te verwaarlozen is (Orgel and Lohrman, 1974).

Tijdens de formose reactie wordt racemisch ribose gevormd. Juist de ongecontroleerde vorming van racemische nucleotiden geeft aanleiding tot grote problemen. De door poly(C) gekatalyseerde oligomerisatie van racemisch $\underline{1}^{A}$ verloopt zeer inefficiënt (Joyce and Visser et al., 1984). Hoewel de inbouw van de L-isomeer langzamer gaat dan van de D-isomeer, belemmert de ingebouwde L-isomeer de vorming van langere ketens. Op een poly(C) matrijs, die uitsluitend D-ribosiden bevat, kan de L-isomeer alleen koppelen met de gevormde oligomeren van D-guanyl zuur als deze L-isomeer de syn-configuratie aanneemt (zie Figuur 1.3). Door deze configuratie gaat de optimale oriëntatie van de nucleofiele 3'-OH van de gekoppelde L-guanyl zuur verloren.

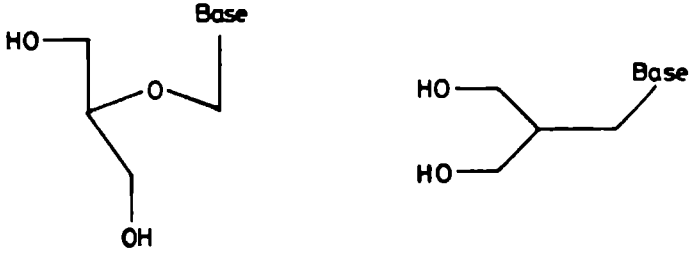
Vanwege de hierboven beschreven moeilijkheden betreffende de vorming van de uitgangsstoffen en de chiraliteit werd er gepostuleerd dat het RNA is voorafgegaan door zich zelf-reproducerende oligomeren die afgeleid zijn van acyclische, prochirale nucleotide-analogen zoals $\underline{6}$ en $\underline{7}$ (Joyce et al., 1987; Joyce, 1989; Orgel, 1987). Hoewel men met de achirale verbindingen van figuur 1.4 het probleem van de door stereoisomeren veroorzaakte remming even zou ontlopen, ontstaan bij oligomerisatie vergelijkbare problemen. Tijdens condensatie reacties van deze prochirale verbindingen moeten atactische oligomeren ontstaan. Daar deze ketens echter een flexibele struktuur bezitten, behoeft de vorming van atactische oligomeren geen belem-



5

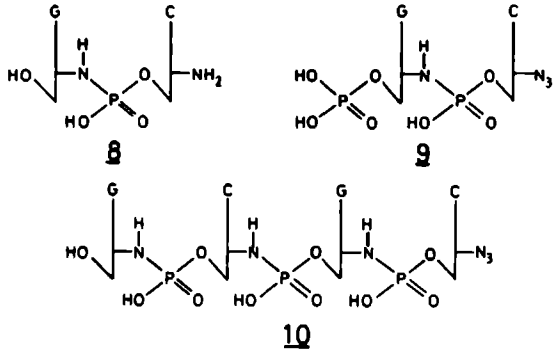
Figuur 1.3: Schematische voorstelling van de syn-configuratie van L-guanyl zuur.

merende werking op de condensatie reacties te hebben (Joyce et al., 1987). In dit verband interessante verbindingen zijn bisfosfaat nucleoside-analogen afgeleid van 6 of 7, die in plaats van de suiker-rest een condensatie produkt tussen formaldehyde en respectivelijk glycerol en acroleïne bezitten (Joyce et al., 1987; Schwartz and Orgel, 1985; Visscher and Schwartz, 1988; Tohidi and Orgel, 1989).



6 7

Figuur 1.4: Acyclische nucleotide analogen waarvan het suiker gedeelte is gebaseerd op de condensatie producten tussen formaldehyde en respectivelijk glycerol (6) en acroleïne (7).

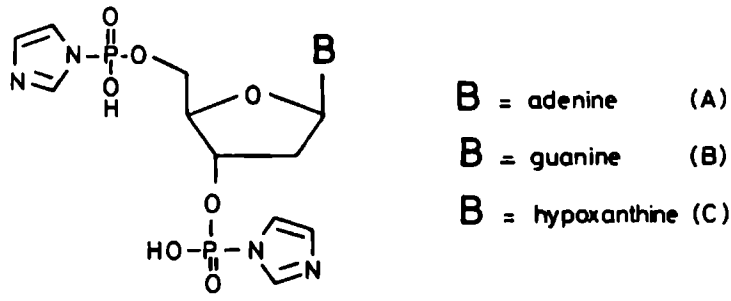


Figuur 1.5: De structuren van fosforamidaten; de dimeren $GNHPCNH_2$ 8 en $PGNHPCN_3$ 9 en de tetrameer $GNHpCNHPGNHPCN_3$ 10

1.4 Het onderzoek

De eerste stap bij het ontwikkelen van een model voor prebiotische replicatie betreft een onderzoek naar de mate waarin men RNA kan modificeren zonder dat de matrijsfunctie verloren gaat. Het is bekend dat enzymatisch gesynthetiseerde polyribonucleotiden reacties kunnen katalyseren, waarbij oligomeren gevormd worden waarin niet de normale 3',5'-fosfodiëster bindingen voorkomen. De synthese van oligo(G), gekatalyseerd door poly(C) en Pb^{2+}-ionen geeft oligomeren met 2',5'-fosfodiësters (Lohrmann and Orgel, 1980). Oligomerisatie reacties van geactiveerd 3'-amino-3'-deoxyguanosine 5'-fosfaat op poly(C) tonen aan dat de fosfodiëster bindingen vervangen kunnen worden door fosforamidaten (figuur 1.5, Zielinski and Orgel, 1985). Voorts werd gedemonstreerd dat fosforamidaat bevattende polynucleotiden in principe als matrijs kunnen dienen door de autokatalytische reproductie van de dimeren 8 en 9 waarbij tetranucleotide-analogen 10 als matrijs fungeerde (Zielinski and Orgel, 1987).

Als uitgangspunt voor het gewenste model hebben wij gekozen voor analogen van DNA waarin een 3',5'-pyrofosfaat rest de normale 3',5'-fosfaat vervangt. Deze analogen kunnen gevormd worden vanuit de geaktiveerde 3',5'-bisfosfodeoxynucleosiden 11, die bij oligomerisatie 3'-3', 3'-5', of 5'-5' pyrofosfaten kunnen vormen (Schwartz



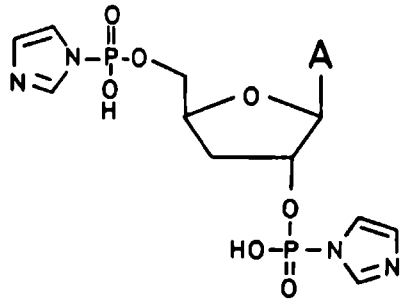
11

Figuur 1.6: De structuren van bisfosfoimidazolaten van $\underline{11}^A$ deoxyadenosine, $\underline{11}^B$ deoxyguanosine, en $\underline{11}^C$ deoxyinosine.

and Orgel, 1985).

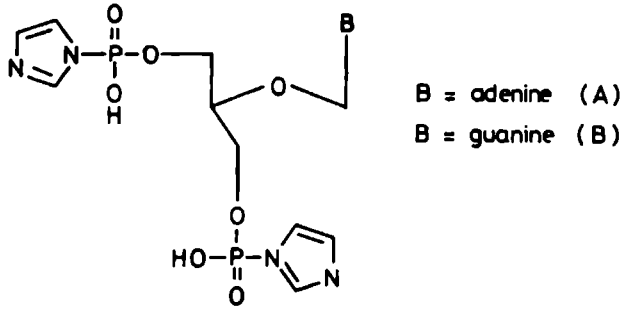
In het tweede hoofdstuk worden de oligomerisatie reacties beschreven van de purine analogen $\underline{11}^A$, $\underline{11}^B$, en $\underline{11}^C$. Door een gunstig combinatie van stackingsinterakties en geometrische oriëntatie vindt juist voor de analogen van adenosine en guanosine een spontane oligomerisatie plaats, waarbij polymeren tot een ketenlengte van twintig nucleotiden gevormd worden. Een complementaire polynucleotide matrijs katalyseert deze reactie, waarbij polymeren met ketenlengtes van langer dan 40 nucleoside resten gevormd worden. Als storende nevenreactie treedt interne cyclisatie op. Door sterische hindering kan deze interne cyclisatie verminderd worden, zoals wordt aangetoond door de oligomerisatie reacties van $\underline{12}$ (hoofdstuk 3). Deze analoog ondergaat spontaan een efficiënte oligomerisatie, waarbij een nieuw type pyrofosfaatbrug ontstaat. Reacties van deoxyadenosine-dimeren met 3'-3', 5'-5', en 3'-5' pyrofosfaat functies laten zien dat een complementaire poly(U) matrijs voorkeur vertoont voor de vorming van 3'-5' pyrofosfaat bindingen (hfd 4).

In hoofdstuk 5 worden de reacties beschreven van geactiveerde acyclische nucleotide analogen $\underline{13}$. In deze nucleotide analogen vervangt een 2-(oxymethyleen)-1,3-propaandiol rest de ribosyl rest. Door de flexibele struktuur is de tendens tot interne cyclisatie sterk, waardoor de spontane oligomerisatie wordt belemmerd. Ondanks dit katalyseert een complementaire polyribonucleotide matrijs de



12

Figuur 1.7: De structuur van bisfosforoimidazolaat van 12 3'-deoxyadenosine.



13

Figuur 1.8: De structuren van bisfosforoimidazolaten van de acyclische analogen $\underline{13}^A$ 2'-nor-2'-deoxyadenosine en $\underline{13}^B$ 2'-nor-2'-deoxyguanosine.

oligomerisatie.

In het zesde hoofdstuk wordt het effect van complexvorming van mangaan-ionen met nucleotide analogen beschreven. Voor de purine analogen wordt de interne cyclisatie geremd, waardoor de spontane oligomerisatie effectiever wordt. Met behulp van mangaan worden lange oligomeren, bestaande uit pyrimidine nucleosiden, door pyrofosfaat resten gekoppeld, van zowel de 2'-deoxyribose- als de acyclische analogen gevormd (hoofdstuk 7).

Oligonucleotide analogen die pyrofosfaat resten bevatten kunnen alleen in aanmerking komen als prebiotische voorlopers, wanneer er een mogelijkheid bestaat tot

hun reproductie. Het vermogen hiertoe hangt af van hun tendens tot complexvorming, die mogelijk wordt gemaakt door base-stacking en vorming van Watson-Crick baseparen van complementaire polymeren. De synthese van oligomeren die pyrofosfaat resten bevatten, blijkt eveneens aan matrijzen te kunnen verlopen, zoals werd aangetoond met enzymatisch verkregen polynucleotiden. Dit bewijst dat de vorming van een complex tussen het polynucleotide en het analoog mogelijk is en in feite optreedt. In hoofdstuk 8 wordt de synthese beschreven van oligomeren van 3'-fosforodeoxycytidyl zuur (pdCp). Oligomerisatie reacties van $\underline{11}^{B}$ in aanwezigheid van oligomeren van pdCp tonen aan dat deze DNA-analogen, die dus pyrofosfaatresten bevatten, de synthese van zijn complementaire vorm katalyseren.

Dit proefschrift heeft een basis gelegd voor toekomstig onderzoek aan enzymvrije reproductie systemen, die in aanmerking zouden kunnen komen als modellen van prebiotische voorlopers van het huidige genetische systeem. Evolutionair gezien is het een aantrekkelijke hypothese, dat het huidige genetische systeem is ontstaan uit primitieve nucleotide analogen, waarin de (2'-deoxy)-D-pentose vervangen is door verbindingen die gevormd zijn onder geologisch aanvaardbare voorwaarden. De niet-enzymatische reproductie van dergelijke moleculen kan echter pas getest worden, wanneer er efficiënte technieken ontwikkeld zijn voor de synthese van isotactische polymeren van deze analogen.

Referenties

Acevedo, O.L., Orgel, L E. (1987) Non-enzymatic transcription of an oligonucleotide 14 residues long. J. Mol. Biol. 197, 187-193.

Bridson, P.K., and Orgel, L.E. (1980) Catalysis of accurate poly(C)-directed synthesis of 3'-5'-linked oligoguanylates by Zn(2+). J. Mol. Biol. 144, 567-577.

Cech, T.R. (1986) A model for the RNA-catalyzed replication of RNA. Proc. Natl. Acad. Sci USA 83, 4360-4363.

Cech, T.R. (1987) The chemistry of self-splicing RNA and RNA enzymes. Science 236, 1532-1539.

Cech, T.R. (1989) Ribozyme self-replication ? Nature 339, 507-508.

Crick, F.H.C. (1968) The origin of the genetic code. J. Mol. Biol. 38, 367-379.

Darnell, J.E., Doolittle, W.F. (1986) Speculations on the early course of evolution. Proc. Natl. Acad. Sci. USA 83, 1271-1275.

Dose, K. (1987) Prabiotische evolution und der ursprung des lebens. Ein kritischer ruckblick. Chemie in unserer Zeit 21.6, 177-185.

Doudna, J., Szostak, J.W. (1989) RNA-catalysed synthesis of complementary-strand RNA. Nature 339, 519-523.

Eigen, M., Schuster, P. (1982) Stages of emerging life. Five principles of early organization. J. Mol. Evol. 19, 47-61.

Fuller, W.D., Sanchez, R.A., Orgel, L.E. (1972) Studies in prebiotic synthesis VII. Solid-state synthesis of purine nucleosides. J. Mol. Evol. 1, 249-257.

Gilbert, W. (1986) The RNA world. Nature 319, 618.

Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., Altman, S. (1983) The RNA moiety of ribonuclease P is the catalytic subunit of the enzym. Cell 35, 849-857.

Heartle, T., Orgel, L.E. (1986) Template-directed synthesis on the oligonucleotide d(C_7-G-C_7). J. Mol. Biol. 188, 77-80.

Hill, A.R., Nord, L.D., Orgel, L.E., Robins, R.K. (1988) Cyclization of nucleotide analogues as an obstacle to polymerization. J. Mol. Evol. 28, 170-171.

Inoue, T., Joyce, G.F., Greskowiak, K., Orgel, L.E. Brown, J.M., Reese, C.B. (1984) Template-directed synthesis on the pentanucleotide CpCpG-pCpCp. J. Mol. Biol. 178, 669-676.

Inoue, T., Orgel, L.E. (1983) A nonenzymatic RNA polymerase model. Science 219, 859-862.

Inoue, T., and Orgel, L.E. (1982) Oligomerization of (guanosine 5'-phosphor)-2-methylimidazolide on poly(C). An RNA polymerase model. J. Mol. Biol. 162, 201-217.

Joyce, G.F. (1989) RNA evolution and the origin of life. Nature 338, 217-224.

Joyce, G.F., Inoue, T., Orgel, L.S. (1984) Non-enzymatic template-directed synthesis on RNA random copolymers. Poly(C,U) templates. J. Mol. Biol. 176, 279-306.

Joyce, G.F., Orgel, L.E. (1986) Non-enzymic template-directed synthesis on RNA random copolymers. J. Mol. Biol. 188, 433-441.

Joyce, G.F., Orgel, L.E. (1988) Non-enzymatic template-directed synthesis on RNA random copolymers. J. Mol. Biol. 202, 677-681.

Joyce, G.F., Visser, G.M., van Boeckel, A.A., van Boom, J.H., Orgel, L.E., Westrenen van, J. (1984) Chiral selection in poly(C)-directed synthesis of oligo(G). Nature 310, 602-604.

Kiedrowski von, G. (1986) Ein selbst replizierendes hexadesoxynucleotid. Angew. Chem. 98, 932-934.

Lewin, R. (1986) RNA catalysis gives fresh perspective on the origin of life. Science 231, 545-546.

Lohrmann, R., Bridson, P.K., and Orgel, L.E. (1980) Efficient metal-ion catalyzed template-directed oligonucleotide synthesis. Science 208, 1464-1465.

Lohrmann, R., and Orgel, L.E. (1978) Template-directed polynucleotide condensation as a model for RNA replication. Origins of Life, 235-244.

Lohrmann, R., and Orgel, L.E. (1980) Efficient catalysis of polycitidylic acid-directed oligoguanylate formation by Pb^{2+}. J. Mol. Biol. 142, 555-567.

Miller, S.L., Orgel, L.E. (1974) The origins of life on the earth. Prentice-Hall, Englewood Cliffs, N.Y.

Naylor, R., Gilham, P.T. (1966) Studies on some interactions and reactions of oligonucleotides in aqueous solution. Biochemistry 5, 2722-2728.

North, G. (1987) Back to the RNA world and beyond. Nature 328, 18-19.

Orgel, L.E. (1968) Evolution of the genetic apparatus. J. Mol. Biol. 38, 381-393.

Orgel, L.E. (1986) RNA catalysis and the origins of life. J. Theor. Biol. 123, 127-149.

Orgel, L.E. (1987) Evolution of the genetic apparatus: A review. Cold

Spring Harbor Symposia on Quantitative Biologie Vol 52, 9-16.

Orgel, L.E., Lohrmann, R. (1974) Prebiotic chemistry and nucleic acids replication. Accounts of Chemical Research 7, 368-377.

Saenger, W. (1984) Principles of nucleic acid structure. Springer-Verlag, New York.

Schwartz, A.W., Orgel, L.E. (1985) Template-directed synthesis of novel, nucleic acid-like structures. Science 228, 585-587.

Sleeper, H.L., Orgel, L.E. (1979) The catalysis of nucleotide polymerization by compounds of divalent lead. J. Mol. Evol. 12, 357.

Sulton, J., Lohrmann, R., Orgel, L.E., Schneider-Bernloehr, H., Weimann, B.J. (1969) Nonenzymatic oligonucleotide synthesis on a polycytidylate template. J. Mol. Biol. 40, 227-234.

Sulton, J., Lohrmann, R., Orgel, L.E., Todd Miles, H. (1968) Nonenzymatic synthesis of oligoadenylates on a polyuridylic acid template. Proc. N.A.S. 59, 726-733.

Tohidi, M., Orgel, L.E. (1989) Some acyclic analoques of nucleotides and their template-directed reactions. J. Mol. Evol. 28, 367-373.

Zaug, A.J., and Cech, T.R. (1986) The intervening sequence RNA of Tetrahymena is an enzyme. Science 231, 470-475.

Zielinski, W.S., and Orgel, L.E. (1985) Oligomerization of activated derivatives of 3'-amino-3'-deoxyguanosine on poly(C) and poly(dC) templates. Nucleic Acids Res. 13, 2469-2484.

Zielinski, W.S., and Orgel, L.E. (1987) Autocatalytic synthesis of a tetranucleotide analogue. Nature 327, 346-347.

Chapter 2

Polynucleotide Analogues as Possible Primitive Precursors of Nucleic Acids

Abstract

Activated derivatives of purine-containing deoxynucleoside bisphosphates spontaneously oligomerize to produce pyrophosphate-linked oligodeoxynucleotide analogues. These analogues are of potential interest as models of primitive, polynucleotide precursors. The efficiency of oligomerization (the bisphosphoimidazolides of deoxyguanosine and deoxyadenosine much greater than the bisphosphoimidazolide of deoxyinosine) appears to reflect a combination of stacking forces and the specific geometric orientations of the stacked units. Under favorable conditions, chain lengths greater than 20 have been obtained for oligomers containing deoxyguanosine bisphosphate in the absence of a template. In the presence of a complementary template, the activated derivatives of the bisphosphate of deoxyguanosine and deoxyadenosine oligomerize much more extensively. These observations suggest the possibility that primitive information transfer might have evolved in much simpler systems and that this function was taken over by polynucleotides at a later stage in evolution.

2.1 Introduction

. We have recently shown (Schwartz and Orgel, 1985a) that a new family of polynucleotide analogues can be synthesized on polynucleotide templates by oligomerization of activated 2'-deoxynucleoside 3',5'-bisphosphates. These products are linked by pyrophosphate groups rather than by phosphodiester linkages. As a first step in investigating the possibility that such molecules or related ones might have been primitive, prebiotic precursors of polynucleotides, we have studied the non-template-directed oligomerization of the activated derivatives ImpdApIm, ImpdGpIm and ImpdIpIm (I in Figure 2.1). In the present paper we report that monomers based on adenosine and guanosine will, under appropriate conditions, oligomerize to produce substantial yields of long oligomers even in the absence of a template. The effects of a number of minerals and amino acid polymers on the oligomerization have also been studied.

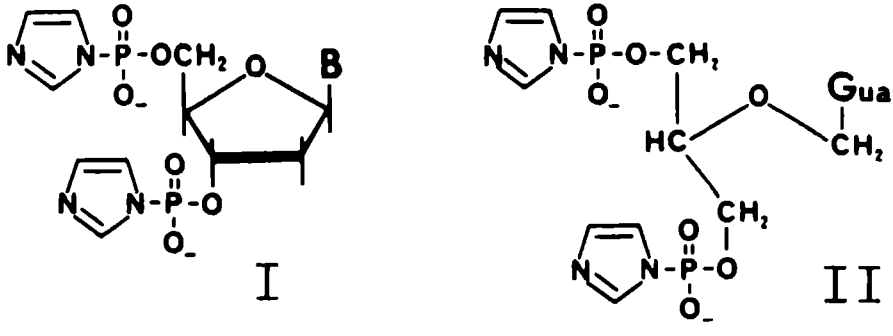


Figure 2.1: Structures of activated monomers. B is adenine, guanine or hypoxanthine.

2.2 Experimental

Deoxynucleosides and polynucleotides, Alkaline Phosphatase (Type III from *E. coli* and Bovine Pancreatic Ribonuclease (Type I-A) were purchased from Sigma Chemical Company. Phosphodiesterase I from *Crotalus adamanteus* venom was purchased

from P-L Biochemicals. The bisphosphates of dG and dI were prepared according to the method of Bennett et al. (1976), except that purification of the products was carried out by chromatography on DEAE-Sephadex (A25, Pharmacia) in the chloride form with a linear gradient of 0.1M to 0.4M NaCl at pH 4.0. Desalting was carried out by adsorbing the diluted fractions on a column in the bicarbonate form and eluting with 1.0M TEAB until no chloride was detectable with $AgNO_3$ and then bringing off the product in 1.0M TEAB. TEAB was removed by repeated evaporation under vacuum in the presence of ethanol. Phosphorylation of dA was performed according to the method of Beld et al. (1984), subsequent to protection of the adenine moiety (Ti et al., 1982). Purification was as described above.

The diimidazolides were prepared by a modification of a standard method used for the preparation of 5'-phosphoimidazolides of nucleotides (Joyce et al., 1984). For pdGp or pdIp (0.6 mmol as triethylamine salts), the bisphosphate was dissolved in 1.3 ml of DMSO plus 0.2 ml of triethylamine. For pdAp, 1.3 ml of DMF plus 0.2 ml of triethylamine was substituted. This solution was added dropwise to 2.5 mmol each of triphenylphosphine, 2',2'-dithiodipyridine (aldrithiol-2) and imidazole, dissolved in 3.3 ml of DMF plus 0.45 ml of triethylamine. Reaction was for 2 h at room temperature under dry conditions, with magnetic mixing. Precipitation of the product was carried out in a dry mixture of 50 ml of acetone, 50 ml of ethyl ether, 4 ml of triethylamine and 0.5 ml of $NaClO_4$-saturated acetone. The products were washed and dried as described in Joyce et al. (1984).

Reaction mixtures were prepared as previously described (Schwartz and Orgel, 1985a) and contained 0.1M diimidazolide, 0.4M $MgCl_2$, 0.1M NaCl, 0.4M imidazole (pH 6.5 with HCl) and, when required, 0.1M poly(U), poly(C), or poly(A). Reaction tubes were allowed to react in a water bath at 4°C for three weeks. At the conclusion of the reaction period, reactions were quenched by addition of 10 μl of 1M KEDTA (pH 9) and 80 μl of H_2O. In some cases poly(U) and poly(C) templates were destroyed prior to HPLC analysis by incubation with ribonuclease.

Enzyme digestions: For Ribonuclease digestion 10 μl of the quenched reaction mixture were added to 100 μl of Tris-HCl (0.05M, pH 7.6) containing 10 units of enzyme. Incubation was at 37°C for 4 h. For Alkaline Phosphatase digestion, 10 μl were added to 100 μl of Tris-HCl (0.04M, pH 8.0) containing 0.02M $MgCl_2$ and 0.1

units of enzyme. Incubation was as above. For Phosphodiesterase-I digestion, 10 μl was added to 100 μl of Tris-HCl (0.2M, pH 9.0) containing 0.04M $MgCl_2$ and 0.2 units of enzyme. Incubation was as above. Before HPLC analysis, 100 μl of sodium acetate (0.1M, pH 4.0) was added to a sample containing 0.1 μmol total nucleotide and the sample was allowed to stand at room temperature overnight to hydrolyze surviving imidazolides.

A number of reactions were also run in the presence of mineral samples or amino acid polymers, to test for the possibility of catalytic effects. Clay Mineral Standards (Ward's) used were kaolinite No. 7; montmorillonites Nos. 23, 24 and 31; and attapulgite No. 43. Other minerals tested included synthetic manganate (gift of G. Arrhenius), production of higher yields of the longest oligomers after extended periods of reaction (Figure 2.2). cacoxenite (gift of P.B. Moore), ferric hydroxide (Schwartz and Orgel, 1985b) and powdered Pueblito de Allende meteorite. The ground minerals (20-30 mg) were washed in imidazole buffer containing magnesium and sodium chlorides at the concentrations to be used in the reactions, which were then carried out as above. After one week, the supernatants were removed by centrifugation in an Eppendorf model 5414 centrifuge at 12 000 rpm and the minerals were extracted with 50 μl of 0.75M $K_4P_2O_7$. To the combined supernatants and extracts was added 10 μl of 1M KEDTA and hydrolysis and analysis were carried out as usual. Polyamino acids were purchased from Sigma. Oligomerizations were conducted in the presence of 0.3M polyaspartic acid, polyglutamic acid and polyarginine. Proteinoids were a gift of S.W. Fox. A series of sixteen proteinoids including "equimolar", "2:2:1", and others including basic, acid and neutral preparations were tested as saturated solutions (for details on the preparation of proteinoids, see Chapter 5 of Fox and Dose, 1977).

2.3 Results and Discussion

It has previously been shown that a number of minerals are without effect on the poly(C)-directed oligomerization of 2-MeImpG (Schwartz and Orgel, 1985b). A large number of mineral samples have now also been tested for their possible effect on the oligomerization of ImpdGpIm in the presence and absence of poly(C), by

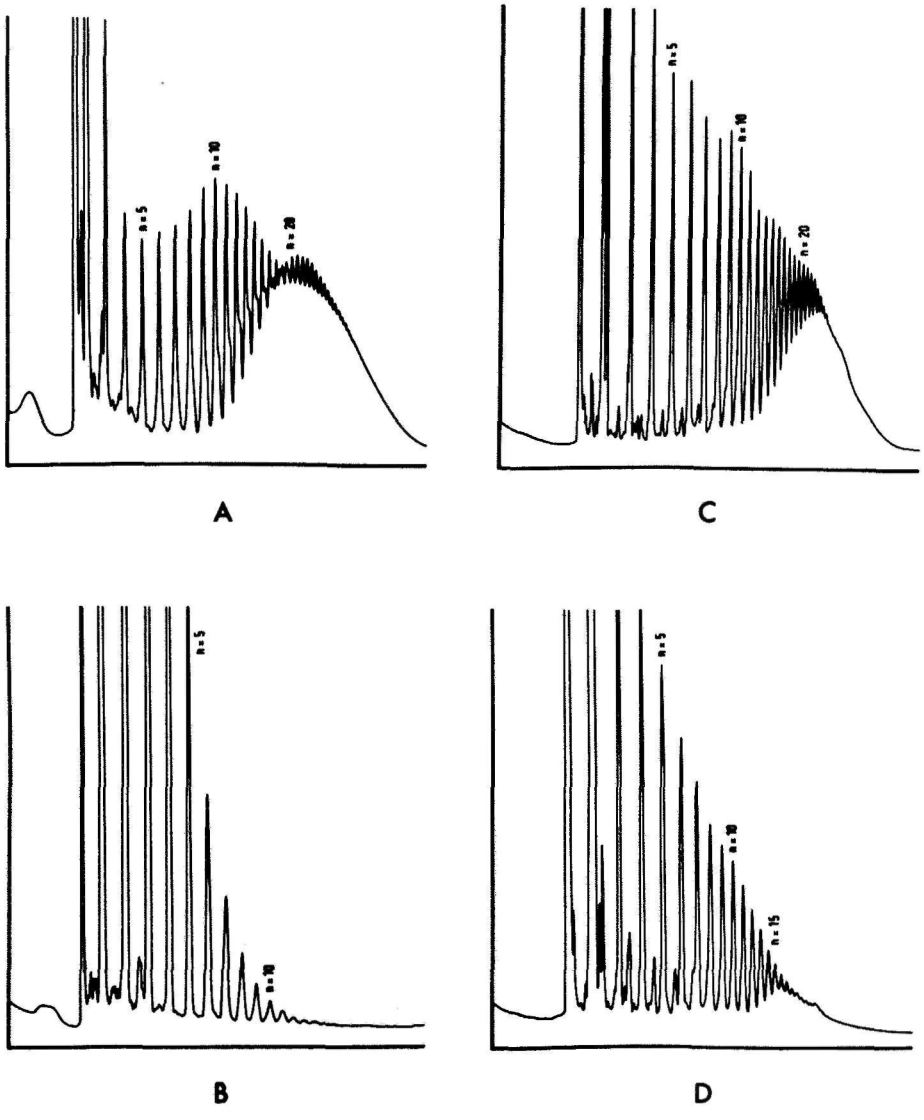


Figure 2.2: Chromatographic analysis of reaction products. **A** ImpdApIm + poly(U); **B** ImpdApIm alone; **C** ImpdGpIm + poly(C); **D** ImpdGpIm alone. For conditions see Experimental.

comparison of the HPLC results with those of control reactions.

Neither stimulation of the reaction nor inhibition were observed for either reaction in the presence of any of the clay minerals, or of hydroxyapatite. Partial inhibition was observed, however, in the presence of manganate and ferric hydroxide. None of the amino acid polymers had a stimulatory effect on the template-free oligomerization, although in some cases a slight inhibition was observed.

HPLC chromatograms of the products of oligomerization reactions are shown in Figure 2.2. After three weeks of reaction, both ImpdApIm and ImpdGpIm produce substantial yields of oligomers. In both template-directed and template-free reactions, the most significant limiting factor is the cyclization of monomer to produce the 3':5' cyclic pyrophosphate. In template-free reactions, 50-60% of the starting material can be consumed in this manner. The cyclization of dimer and trimer appear to be much slower reactions. After three weeks reaction, only about as much cyclic oligomer has accumulated as the remaining linear product (these products can be resolved under slightly different chromatographic conditions). The identities of the cyclic products were established by demonstrating resistance to bacterial alkaline phosphatase and by incubation with venom phosphodiesterase and observing the sequential production (for example for trimer) of linear trimer, dimer and finally the monomer.

The most efficient oligomerizations in the absence of a template were obtained with ImpdGpIm, which gave conversions of monomer to oligomer of 40%. Of these products 18% had chain lengths of 10 or more. In the presence of a complementary template, oligomerization competes favorably with cyclization and oligomer yields in excess of 60% have been obtained. Although template-directed reactions are substantially faster during the first few days of reaction, the most obvious effect of the presence of a complementary template on the oligomerizations of ImpdApIm and ImpdGpIm is the In contrast to ImpdGpIm and ImpdApIm, ImpdIpIm reacted inefficiently. A 33% total yield of oligomers was obtained after three weeks, but no products with chain lengths higher than 8 could be detected (Figure 2.3). Attempts to improve the reaction by addition of a "template" consisting of poly(A) were unsuccessful [The wobble-pair A:I has been suggested as the basis for a hypothetical primitive genetic code (Orgel, cited in Crick, 1968)].

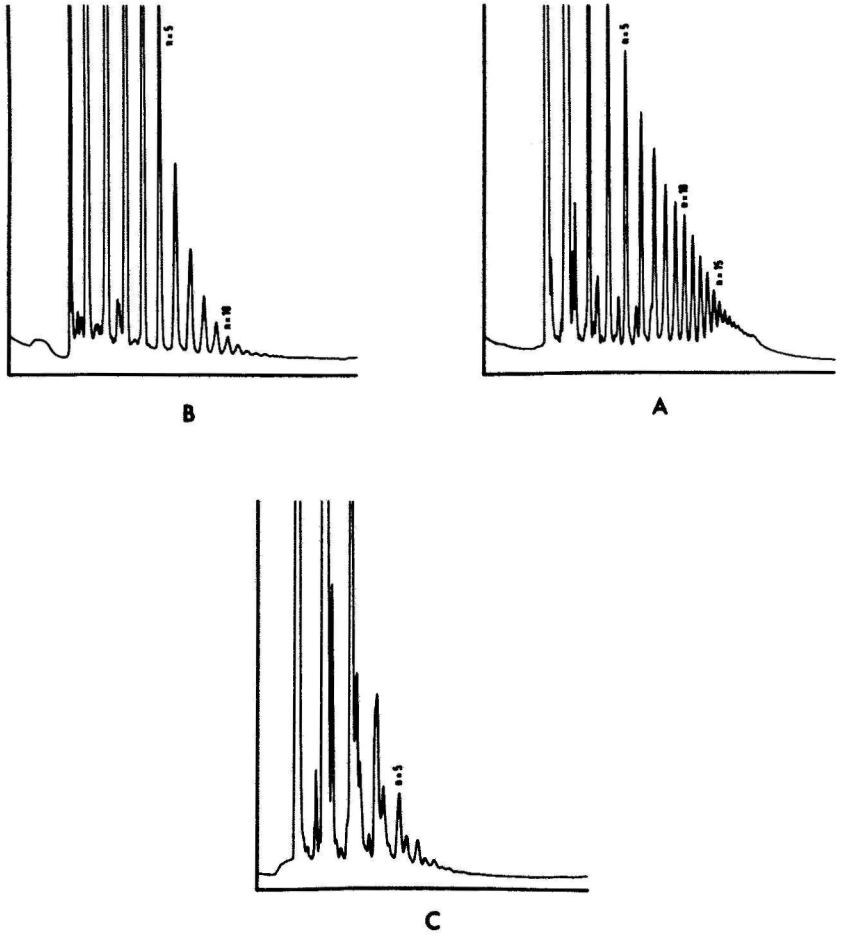


Figure 2.3: Chromatographic analyses of reaction products. **A** ImpdGpIm; **B** ImpdApIm; **C** ImpdIpIm. For conditions see Experimental.

The inefficient oligomerization of ImpdIpIm is unexpected. We believe that stacking plays an important role in permitting the formation of long oligomers in the absence of a complementary template. pI is known to stack at least as strongly in aqueous solution as pG, although much less strongly than pA (Neurohr and Mantsch, 1979). A possible explanation for our results may be the known aggregation of guanosine nucleotides to form stacked, H-bonded tetramers (Bouhoutsos-Brown et al., 1982). Monomers participating in such aggregates might be constrained to orientations with O-6 directed inward (relative to the tetramer), thereby discriminating against head to head stacking of monomers. Although tetramers might well be stacked head to head, individual deoxyribose units would still be in position to permit pyrophosphate formation. In support of this hypothesis, we have observed that the addition of potassium ions to reaction mixtures enhances the production of higher oligomers from ImpdGpIm (Bouhoutsos-Brown et al., 1982) but not from ImpdApIm or ImpdIpIm.

We have earlier reported that the dimonophosphate of the acyclic nucleoside analogue 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (II in Figure 2.1) can also be oligomerized on a poly(C) template (Schwartz and Orgel, 1985a). Compounds of this type are interesting since - being initially achiral - they can be regarded as possible precursors of chiral polynucleotides (Joyce et al, 1987). In order to test the hypothesis that nucleic acid analogues with novel backbones might have played a role in chemical evolution, it will be necessary to synthesize and test such oligomers as templates.

References

Beld, A., Claesen, C.A.A., Roersma, E.S., Schipper, W.J.M., Keiser, L.M., Tesser, G.I. (1984) Bis [2-(methylsulfonyl)ethyl] phosphorochloridate, a new phosphorylating agent. Recl. Trav. Chim. Pays-Bas 103, 196-202.

Bennett, G.N., Gough, G.R., Gilham, P.T. (1976) Guanosine tetraphosphate and its analogues. Chemical synthesis of guanosine 3',5'-dipyrophosphate deoxyguanosine 3',5'-dipyrophosphate, guanosine 2',5'-bis(methylenediphosphonate), and guanosine

3',5'-bis(methylenebisphosphonate). Biochemistry 15, 4623-4628.

Bouhoutsos-Brown, E., Marshall, L., Pinnavaia, T.J. (1982) Structure-directing properties of Na^+ in the solution ordering of guanosine 5'-monophosphate. Stoichiometry of aggregation, binding to ethidium, and modes of Na^+ complexation. J. Am. Chem. Soc. 104, 6576-6584.

Crick, F.H.C. (1968) The origin of the genetic code. J. Mol. Biol. 38, 367-379.

Fox, S.W., Dose, K. (1977) Molecular evolution and the origin of life. Marcel Dekker Inc., New York.

Joyce, G.F., Inoue, T., Orgel, L.S. (1984) Non-enzymatic template-directed synthesis on RNA random copolymers. J. Mol. Biol. 176, 279-306.

Joyce, G.F., Schwartz, A.W., Miller, S.L., Orgel, L.E. (1987) The case for an ancestral genetic system involving simple analogues of the nucleotides. Proc. Natl. Acad. Sci. USA 84, 4398-4402.

Neurohr, K.J., Mantsch, H.H. (1979) The self-association of naturally occurring puring nucleoside 5'-monophosphates in aqueous solution. Canadian J. Chem. 57, 1986-1994.

Schwartz, A.W., Orgel, L.E. (1985a) Template-directed synthesis of novel, nucleic acid-like structures. Science 228, 585-587.

Schwartz, A.W., Orgel, L.E. (1985b) Template-directed polynucleotide synthesis on mineral surfaces. J. Mol. Evol. 21, 299-300.

Ti, G.S., Gaffney, B.L., Jones, R.A. (1984) Transient protection. Efficient one-flask synthesis of protected deoxynucleotides. J. Am. Chem. Soc. 104, 1316-1319.

Chapter 3

Oligomerization of 3'-Deoxyadenosine 2',5'-Bisphosphoimidazolide

Abstract.

The activated bisphosphate of 3'-deoxyadenosine (cordycepin) undergoes oligomerization to produce a new family of pyrophosphate-linked oligomers in which the average repeating unit involves a nine-atom structural group. The presence of a poly(U) template increases the relative yields of higher oligomers, although the template-free reaction is itself extremely efficient.

3.1 Introduction

The template-directed synthesis of a new family of pyrophosphate-linked, nucleic acid analogues has been described (Schwartz and Orgel, 1985). Recently we have presented additional data on the non-template-directed (spontaneous) oligomerizations of the 3',5'-bisphosphoimidazolides of 2'-deoxyguanosine, 2'-deoxyadenosine, and 2'-deoxyinosine (Schwartz et al., 1987). These oligomerizations represent a first step toward the development of models of possible primitive precursors of the nucleic acids. Bisphosphorylated deoxymononucleotides are somewhat more plausible than selectively monophosphorylated ribonucleosides. More importantly, oligomerizations of the activated forms of these monomers can be carried out in admixture with other monomers to produce mixed products, without seriously inhibiting the reaction (Schwartz and Orgel, 1985). The extension of these oligomerizations to

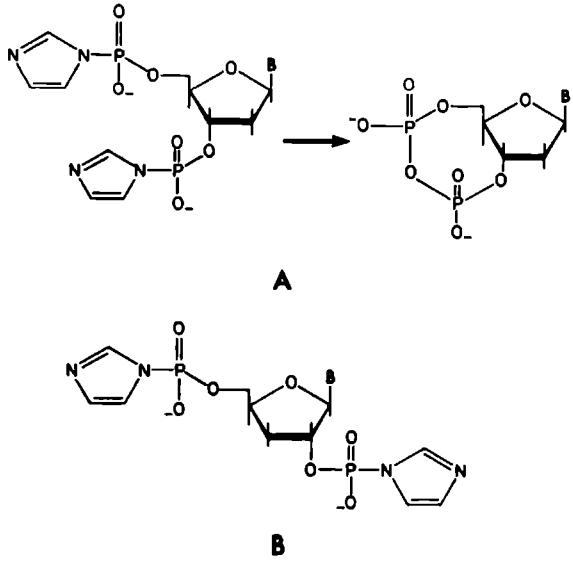


Figure 3.1: **A** Cyclization of a 2'-deoxynucleoside 3',5'-bisphosphoimidazolide; **B** the structure of a 3'-deoxynucleoside 2',5'-bisphosphoimidazolide.

acyclic analogues of nucleosides provides the possibility of an even greater extrapolation toward more primitive ancestors of contemporary nucleic acids (Joyce et al.,

1987).

A limiting factor in these reactions is the competing cyclization of the monomer bisphosphoimidazolides to produce 3',5'-cyclic pyrophosphates (Fig. 3.1^A). Cyclization often consumes 50% of the starting material in spontaneous reactions. Since cyclization of a 3'-deoxynucleoside 2',5'-bisphosphate (Fig. 3.1^B) is expected to be limited by steric hindrance, we have synthesized 3'-deoxyadenosine 2',5'-bisphosphoimidazolide and studied its oligomerization in the presence and absence of the complementary template poly(U).

3.2 Experimental

Adenosine, poly(U), alkaline phosphatase (type III from *E.coli*), and bovine pancreatic ribonuclease (type I-A) were purchased from Sigma Chemical Company. Phosphodiesterase I from *Crotalus adamanteus* venom was purchased from P-L Biochemicals. Cordycepin (3'-deoxyadenosine) was prepared following the method of Hansske and Robins (1985). After protecting the adenine moiety (Ti et al.), the 2',5'-bisphosphate was prepared according to the method of Beld et al. (1984) and purified by chromatography on DEAE-Sephadex (A25, Pharmacia) with a linear gradient of 0.1M to 0.6M TEAB/2000ml at pH 8.0. TEAB was removed by repeated evaporation under vacuum in the presence of ethanol. The diimidazolide [Impd(3')ApIm] was prepared as previously described for ImpdApIm (Schwartz et al., 1987).

Reaction mixtures were prepared as described by Schwartz and Orgel (1985). The mixtures contained 0.1M Impd(3')ApIm or ImpdApIm, 0.2M $MgCl_2$, 0.1M NaCl, 0.4M Bis-Tris HCl (pH 6.5) and when required 0.1M (monomer-equivalent) poly(U). The reactions were maintained for periods varying from one day to three weeks at a temperature of 4°C. At the conclusion of this period, reactions were quenched by addition of 2.5 μl of KEDTA (1.0M, pH 9.0) and 7.5 μl of $K_4P_2O_7$ (1.0M, pH 9.0) was added to desorb products and template from the glass. The poly(U) was destroyed by incubation with pancreatic ribonuclease [1 μl of quenched reaction mixture was added to 100 μl of Tris-HCl, (0.05M, pH 7.6) containing 10 units of enzyme. Incubation was for 12 h at 37°C]. Surviving imidazolides were

destroyed by hydrolysis at pH 4.0 [1 μl of reaction mixture was added to 100 μl of 0.1M sodium acetate, pH 4.0, and heated at 50°C for 4h].

Analysis was by chromatography on RPC-5 in 0.02M NaOH with a linear gradient of $NaClO_4$ (0-0.04M over 60 min) at a flow rate of 1.0 ml/min. Peak detection was by absorbance monitoring at 254 nm. Identification of individual oligomers, which were collected from the RPC-5 column and desalted on DEAE-Sephadex (Schwartz et al., 1987), was aided by degradation with phosphodiesterase. Thus a desalted oligomer fraction, containing 0.2 ODU of total nucleotide was added to 60 μl of Tris-HCl (0.2M, pH 9.0) containing 0.04M $MgCl_2$ and 0.015 units of enzyme. Samples were incubated for 2.5, 5, 20 and 70 min and were analyzed by HPLC on RPC-5. Degradation of the pentamer, for example, gave tetramer, trimer, dimer and monomer. Identification of the cyclic monomeric pyrophosphate, which is difficult to separate from monomer on RPC-5, was aided by digestion with alkaline phosphatase. Monomer is degraded to nucleoside by this treatment while the cylic pyrophosphate is unaffected. One μl of quenched reaction mixture was added to 100 μl of Tris-HCl (0.04M, pH 8.0) containing 0.02M $MgCl_2$ and 0.1 units of enzyme. Incubation was for 4 h at 37°C.

3.3 Results and Discussion

The overall rate of oligomerization of Impd(3')ApIm was surprisingly high. After a reaction period of one day, 52% of the starting material had reacted to form dimer and higher products in the absence of poly(U). In the presence of poly(U), a 65% yield was obtained. In both cases only a trace of a peak with properties corresponding to the cyclic pyrophosphate was detected. These total conversions of monomer after a reaction period of one day are actually greater than those obtained with ImpdApIm after three weeks [43% in the presence of poly(U)], although the distributions of chain lengths obtained display a different pattern (see below). The relatively rapid oligomerization of the 3'-deoxy isomer is most likely a result of the availability of higher average concentrations of monomer, due to inhibition of the competing cyclization process. Molecular models suggest that only a highly distorted ribofuranose ring can accommodate cyclic pyrophosphate-bond formation. Ikehara and

Table 3.1: Product distributions in the oligomerizations of ImpdApIm and Impd(3')ApIm*

Reaction	Conversion of monomer to oligomers (%, $n \geq 2$)	Cyclization of monomer (%)	Unreacted monomer (%)	Relative yields of oligomers of length n (%)	
				$n \geq 5$	$n \geq 10$
ImpdApIm	33	43	20	6	0.5
ImpdApIm + poly(U)	43	45	7	30	20
Impd(3')ApIm	80	3	17	24	1
Impd(3')ApIm + poly(U)	86	2	12	35	10

* For reaction conditions see Experimental.

Yano (1974) were able to synthesize cordycepin 2':5'-cyclic phosphodiester, but only via a route involving the cyclization of 8,3'-S-cycloadenosine 5'-phosphate, which is known to have an extreme 3'-endo conformation (Tomita et al., 1972). Treatment of cordycepin 5'-phosphate with DCC in anhydrous pyridine gave a 5.7% yield of the cyclic ester. Presumably, conditions in aqueous solution are too unfavorable to permit cyclization of Impd(3')ApIm.

Fig. 3.2 compares the patterns of products obtained in the oligomerization in the presence and absence of poly(U) after three weeks. The total conversion of monomer to oligomers was 80% in the absence of a template, with 24% of the oligomers having chain lengths of five or more. In the presence of poly(U) the yield was 86% and 35% of the oligomers were of pentamer and longer length. (See Table 3.1). These differences, while significant, are less dramatic than those observed in conventional template-directed oligomerizations of 2-methylimidazolides of ribonucleoside 5'-phosphates (Inoue and Orgel, 1983), or in the template-directed oligomerizations of 3',5'-bisphosphoimidazolides of 2'-deoxynucleosides (Schwartz et al., 1987). In both of these latter classes of oligomerization, the effect of the template in increasing the relative yields of the longest oligomers is more pronounced. Since the catalytic effect of a complementary template is attributable to the formation of a helical complex, the interaction between a polyribonucleotide (a six-atom structural unit) and the complementary 2',5'-pyrophosphate-linked oligomer (a nine-atom structural unit) may be less stable than either of the above systems.

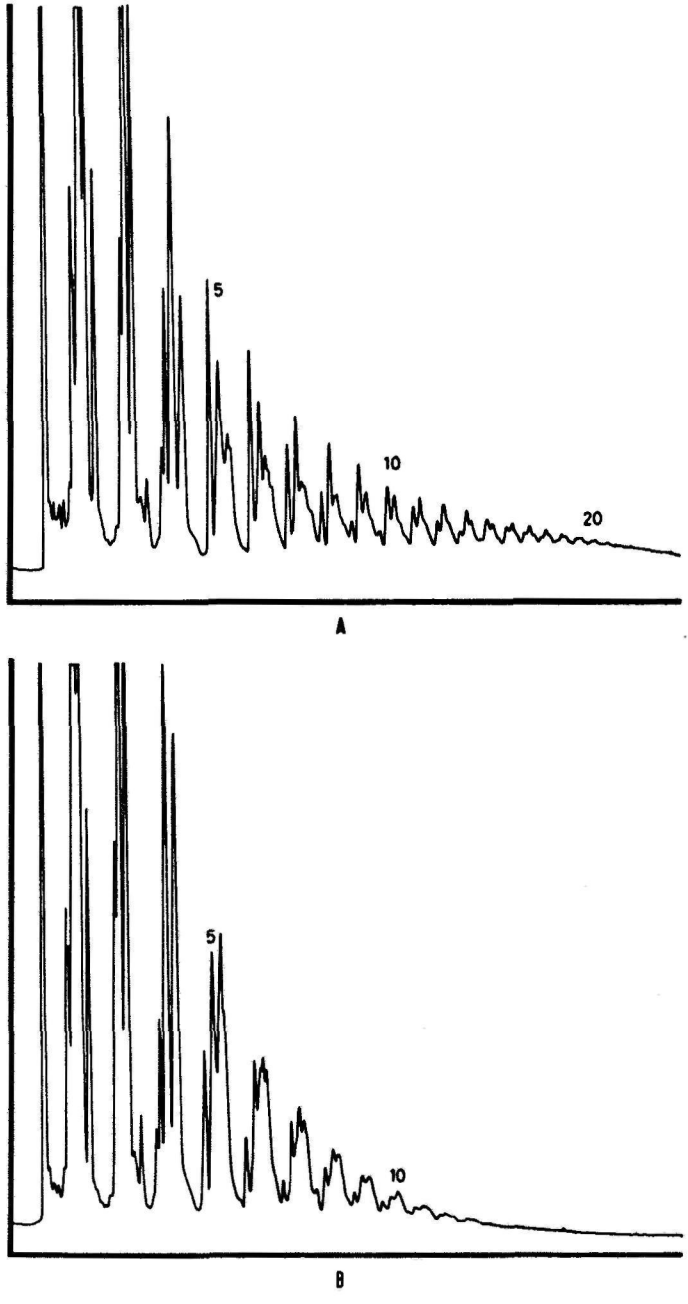


Figure 3.2: Chromatographic analysis of reaction products. **A** Impd(3')ApIm + poly(U); **B** Impd(3')ApIm alone. For conditions see Experimental.

Indications have been detected in the past that the products of spontaneous oligomerizations of activated 2'-deoxynucleoside 3',5'-bisphosphates constitute a complex mixture of linkage types (Schwartz and Orgel, 1985). The 3'-deoxy analogues, however, seem to be much better resolved on the RPC-5 column. The complex nature of the sets of peaks in Fig. 3.2 is probably due to the occurrence of various modes of linkage (That all oligomers are indeed pyrophosphate-linked is supported by degradation with phosphodiesterase I, which produces a series of oligomers of decreasing length and ultimately, the monomer). The differences in the patterns of peaks seen after reaction with or without poly(U) may be due to a limited degree of regiospecific control by the template.

References

Beld, A., Claesen, C.A.A., Roersma, E.S., Schipper, W.J.M., Keiser, L.M., Tesser, G.I. (1984) Bis [2-(methylsulfonyl)ethyl] phosphorochloridate, a new phosphorylating agent. Recl. Trav. Chim. Pays-Bas 103, 196-202.

Hansske, F., Robins, M.J. (1985) Regiospecific and stereoselective conversion of ribonucleosides to 3'-deoxynucleosides. A high yield three-stage synthesis of cordycepin from adenosine. Tetrahedron Lett. 26, 4295-4298.

Ikehara, M., Yano, J. (1974) Seven-membered cyclic phosphate: synthesis and properties of S-cycloadenosine 2',5'-cyclic phosphate and cordycepin 2',5'- cyclic phosphate. Studies of nucleosides and nucleotides. LXIV. Nucleic Acids Res. 1, 1783-1798.

Inoue, T., Orgel, L.E. (1983) A nonenzymatic RNA polymerase model. Science 219, 859-862.

Joyce, G.F., Schwartz, A.W., Miller, S.L., Orgel, L.E. (1987) The case for an ancestral genetic system involving simple analogues of the nucleotides. Proc. Natl. Acad. Sci. U.S.A. 84, 4398-4402.

Schwartz, A.W., Orgel, L.E. (1985) Template-directed synthesis of novel, nucleic acid-like structures. Science 228, 585-587.

Schwartz, A.W., Visscher, J., Bakker, C.G., Niessen, J. (1987) Nucleic

acid-like structures II. Polynucleotide analogues as possible primitive precursors of nucleic acids. Origins of Life 17, 351-357.

Ti, G.S., Gaffney, B.L., Jones, R.A. (1984) Transient protection. Efficient one-flask synthesis of protected deoxynucleotides. J. Am. Chem. Soc. 104, 1316-1319.

Tomita, K., Tanaka, T., Yoneda, M., Fujiwara, T., Ikehara, M. (1972) Crystal and molecular structure of S-cycloadenosine derivatives, 8,3'-S-cycloadenosine and 8,2'-S-cycloadenosine-3',5'-cyclic monophosphate. Acta Crystallog. A28, S45.

Chapter 4

Oligomerization of Deoxynucleoside-Bisphosphate Dimers: Template and Linkage Specificity

Abstract

Evidence is presented that a poly(U) template selectively favors the oligomerization of the activated, 3'-5' pyrophosphate-linked dimer pdAppdAp, in comparison with the 3'-3' and 5'-5' linked dimers. In the absence of poly(U), the 5'-5' linked dimer is the most reactive, and chains are formed which are more than 60 monomer units in length.

4.1 Introduction

2'-Deoxynucleoside 3',5'-bisphosphoimidazolides containing adenine or guanine oligomerize to produce long, pyrophosphate-linked oligomers in the presence of the complementary polynucleotide template (Schwartz and Orgel, 1985). In the absence of a template, oligomerization still occurs, but is much less efficient (Schwartz et al., 1987). In order to provide information on the ability of the polynucleotide template to direct the mode of linkage of the products, we have synthesized the 3'-3', 5'-5' and 3'-5' linked dimers of pdAp and investigated their oligomerization in the presence and in the absence of a poly(U) template.

4.2 Experimental

Alkaline phosphatase (type III from*E. Coli*), bovine pancreatic ribonuclease (type I-A), and poly(U) were purchased from Sigma Chemical Company. EDAC was purchased from Janssen Chimica The dimers of pdAp linked by 3'-3', 3'-5' or 5'-5' pyrophosphate bonds were synthesized as described by Woerd et al. (1987). Imidazolation of the free phosphates of the dimers was carried out in solution as follows. Dimers (0.05M) were reacted with 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDAC, 1.34M) and imidazole (1,34M) at pH 6.5 and 0°C. After two hours the formation of the bisphosphoimidazolides was at least 90% complete, as determined by HPLC and ^{31}P-NMR (^{31}P-NMR shifts relative to phosphoric acid in D_2O: 3'-5' $(pdAp)_2$: 3.5, 3.2, -8.6, and -9.5 ppm; bisphosphoimidazolide of 3'-5' $(pdAp)_2$: -6.4, -7.3, -9.5 and -10.0). After activation, 2.5 μl portions of this solution were added to Pyrex tubes containing 2 μmol $MgCl_2$, 1 μmol NaCl and, when required, 0.5 μmol poly(U) (monomer-equivalents). Water was added to produce a final volume of 10 μl. The final concentrations were 0.0125M dimer-bisphosphoimidazolide, 0.2M $MgCl_2$, 0.1M NaCl, approximately 0.3 imidazole and, if added, 0.05M poly(U) (monomer-equivalents). Reactions were carried out at 4°C and pH 6.5 for two weeks. At the conclusion of the reaction, 2.5 μl of KEDTA (1M, pH 9.0) and 7.5 μl of $K_4P_2O_7$ (1M, pH 9.0) were added and the reaction mixture was stored at -25°C. Analytical techniques have been described previously (Schwartz et

Table 4.1: Product distributions in the oligomerizations of dimers of pdAp*.

Dimer	Template	Cyclization of dimers (%)	Yields of Higher Oligomers of length n (%)		
			$n \geq 4$	$n \geq 10$	$n \geq 20$
3'-5'	poly(U)	13	84	69	37
	-	74	21	6	1
5'-5'	poly(U)	41	55	34	12
	-	68	30	19	13
3'-3'	poly(U)	42	53	34	10
	-	73	26	13	6

* For reaction conditions see Experimental.

al., 1987). Thus, prior to analysis poly(U) was destroyed by digestion with pancreatic ribonuclease, and surviving imidazolides were destroyed by hydrolysis at pH 4.0. Analysis was by HPLC on RPC-5 in 0.02M NaOH with a linear gradient of $NaClO_4$ (0 to 0.04M over 60 min) at a flow rate of 1.0 ml/min. Peak detection was by absorbance monitoring at 254 nm. Identification of the cyclic dimers was aided by treatment with alkaline phosphatase. Identification of other oligomers of pdAp was aided by coinjection with previously identified oligomers obtained from ImpdApIm.

4.3 Results and Discussion

Of the three dimers of pdAp studied, only the 3'-5' linked molecule showed a dramatic template effect (Fig. 4.1 and Table 4.1). Both overall yields as well as the production of the longest oligomers were markedly stimulated by the presence of poly(U). Although the 3'-3' and 5'-5' isomers showed some increase in yields in the presence of template, the effect was largely confined to oligomers less than 20 monomer units in length. The oligomerization in the absence of template was very different. The longest oligomers as well as the highest overall conversions of dimer to oligomers were produced in the order: 5'-5' > 3'-3' > 3'-5'.

Remarkably, the presence of the template seemed to *inhibit* production of the

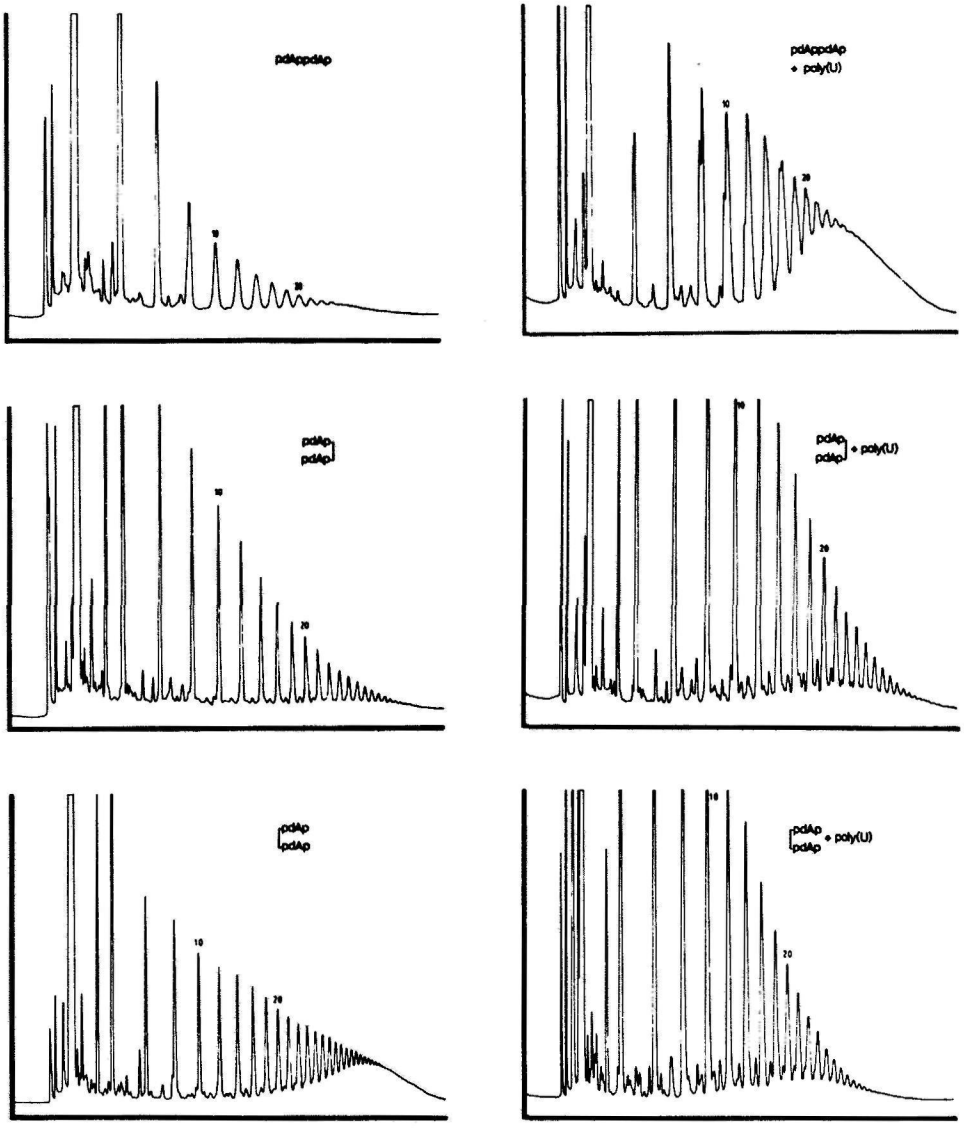


Figure 4.1: HPLC separations of the products of oligomerizations of 3'-5', 3'-3' and 5'-5' linked dimers of pdAp, after activation by coversion to diimidazolides. For conditions and product distributions see Experimental.

longest oligomers from the 5'-5' isomer. It seems a reasonable hypothesis that the most stable structure formed by template and product oligomers is that in which the axial distance between base-pairs along the chain remains constant. The 3'-5' linked dimer can be ordered in this manner by the template. The 3'-3' and 5'-5' dimers, however, can obviously only form staggered structures. In line with this reasoning, it is likely that the strong enhancement in yields of the longest oligomers that is caused by the presence of a poly(U) template in the oligomerization of the monomer ImpdApIm, also reflects the preferred formation of 3'-5' linkages (Schwartz et al., 1987).

The self-organizing properties of the 5'-5' dimer of pdAp are quite remarkable. It should be noted that in spite of the low dimer concentration of 0.0125M, at which cyclization is strongly favored above oligomerization, the reaction in the absence of template produced oligomers with chain lengths exceeding 60 monomeric units.

References.

Schwartz, A.W., Orgel, L.E. (1985) Template-directed synthesis of novel, nucleic acid-like structures. Science 228, 585-587.

Schwartz, A.W., Visscher, J., Bakker, C.G., Niessen, J. (1987) Nucleic acid-like structures II. Polynucleotide analogues as possible Primitive Precursors of nucleic acids. Origins of Life 17, 351-357.

Visscher, J., Schwartz, A.W. (1988) Template-directed synthesis of acyclic oligonucleotide analogues. J. Mol. Evol. 28, 3-6.

Woerd, R. van der, Bakker, C.G., Schwartz, A.W. (1987) Synthesis of P1,P2-dinucleotide pyrophosphates. Tett. Lett. 28, 2763-2766.

Chapter 5

Template-Directed Synthesis of Acyclic Oligonucleotide Analogues

Abstract

Bisphosphoimidazolides of an analogue of adenosine (in which ribose is replaced by an acyclic chain) and of two related analogues of guanosine, undergo oligomerization in the presence of complementary polynucleotide templates. Data on the template and non-template directed reactions are presented, and the possible relevance to origins of life is discussed.

5.1 Introduction

Recently, attention has been called to the possibility that simpler analogues of the polynucleotides may have preceded the first RNA molecules in the course of prebiotic evolution (Schwartz and Orgel, 1985; Schwartz et al., 1987; Joyce et al., 1987). There have been reports of template-directed oligomerizations producing pyrophosphate-linked analogues (Schwartz and Orgel, 1985; Schwartz et al., 1987; Visscher and Schwartz, 1988) as well as phosphoramidate-linked products (Zielinski and Orgel, 1985 and 1987). In both of these cases, use has been made of modified ribo- or deoxyribonucleosides as starting materials. The difficulties presented by the problem of the prebiotic synthesis of ribose and its nucleosides, together with the inhibition of template-directed syntheses observed in the presence of a racemic mixture of mononucleotides (Joyce et al, 1984), make it seem unlikely that the first self-replicating systems made use of the same materials. It has been suggested that contemporary nucleotides were possibly preceded in evolution by primitive ancestors which were flexible, acyclic, prochiral nucleotide analogues (Joyce et al., 1987). An example of such a monomer is 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine ($\tilde{G}$). Preliminary results of the template-directed oligomerization of the bisphosphoimidazolide of ($\tilde{G}$) were presented by Schwartz and Orgel (1985). We now present a more detailed study of the oligomerization reactions of the bisphosphoimidazolides of 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (Imp$\tilde{G}$pIm), 9-[(1,3-dihydroxy-2-propoxy)methyl]adenine (Imp$\tilde{A}$pIm), and of a new analogue, (R)-9-[1-(1-hydroxy-2-propoxy)-2-hydroxyethyl]guanine (Imp$\bar{G}$pIm). See Fig. 5.1 for the structures of these derivatives.

5.2 Experimental

Adenine, guanine, alkaline phosphatase (type III from *E. coli*), bovine pancreatic ribonuclease (type I-A), poly(U), and poly(C) were purchased from Sigma Chemical Company. Phosphodiesterase I from *Crotalus adamanteus* venom was purchased from P-L Biochemicals. EDAC was purchased from Janssen Chimica. 9-[(1,3-Dihydroxy-2-propoxy)methyl]guanine was prepared following the method of

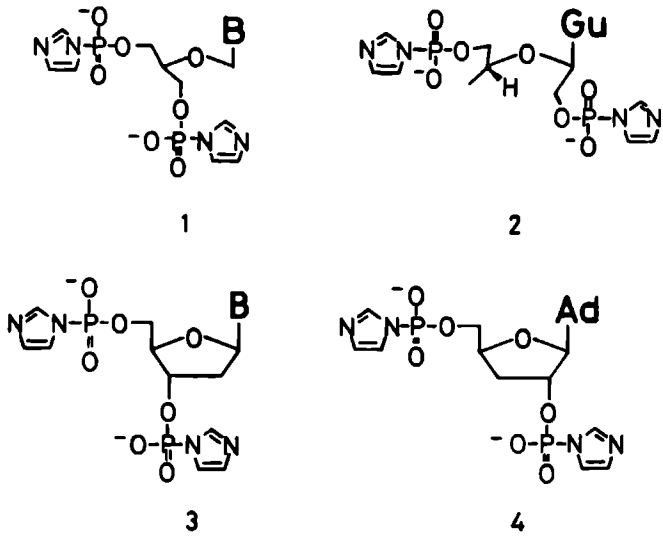


Figure 5.1: Structures of bisphosphoimidazolides (B = adenine or guanine). **1** ImpÃpIm or ImpG̃pIm; **2** ImpḠpIm; **3** ImpdApIm or ImpdGpIm; **4** Imp(3')dApIm.

Ogilvie et al. (1982), by condensing silylated guanine with 1,3-dibenzyloxy-2-chloromethoxypropane. The adenine analogue was similarly prepared by using silylated N-6-benzoyladenine. (R)-9-[1-(1-hydroxy-2-propoxy)-2-hydroxyethyl]guanine was synthesized according to the method of McGee and Martin (1986). The 3',5'- or 2',3'-bisphosphates of the three analogue nucleosides were prepared by the following procedure (von Kiedrowski, personal communication): 1 mmole of the compound was suspended in trimethylphosphate (4 ml). Phosphorylchloride (1 ml) was added dropwise to the suspension at a temperature of 0°C. After 1 h the reaction mixture was frozen in dry ice/acetone and treated with 100 ml of 1M TEAB (pH 7.5). After evaporation to dryness the products were purified as described in Schwartz et al. (1987).

The bisphosphoimidazolides of G̃, Ã, and Ḡ were prepared by treating the acyclic nucleoside bisphosphate (0.1M) with imidazole (1.0M) and EDAC (1.0M) at pH 6.5 and 0°C. After 2 h the reaction was 90% complete and no more than 6% of the cyclic pyrophosphate had formed as determined by HPLC and ^{31}P-NMR (chemi-

cal shifts in D_2O with phosphoric acid as reference: terminal phosphate, 2.7 ppm; phosphoimidazolide, -5.7 ppm; cyclic pyrophosphate, -8 to -11 ppm). Samples (5 μl) were taken from this solution and added to tubes containing 5 μl water, 1 μmol $MgCl_2$, 1 μmol NaCl, and when required 1.0 μmol (monomer-equivalents) of the complementary ribonucleotide template. The final oligomerization mixtures therefore contained 0.05 M bisphosphoimidazolide, 0.1M $MgCl_2$, 0.1M NaCl and, when present, 0.1M of the complementary template in a total volume of 10 μl and a pH of 6.5 (0.4M imidazole as buffer). The reactions were carried out for 7 days at a temperature of 4°C and were quenched by adding 2.5 μl KEDTA (1M, pH 9) and 7.5 μl $K_4P_2O_7$ (1M, pH 9). Storage was at -25°C. Before analysis, any surviving imidazolides and template, if present, were destroyed as described by Schwartz et al. (1987). Analysis was by HPLC on RPC-5 in 0.02M NaOH with a linear gradient of $NaClO_4$ (0-0.04M over 60 min) at a flow rate of 1.0 ml/min. Peak detection was by monitoring absorbance at 254 nm.

Treatment of the reaction mixture with alkaline phosphatase and phosphodiesterase was performed as described by Schwartz et al. (1987). Identification of the peaks corresponding to the cyclic pyrophosphates of monomer and dimer was based on the resistance of these peaks to alkaline phosphatase and conversion to the linear compounds upon treatment with venom phosphodiesterase. The assignment of chain lengths of oligomers was checked by isolating selected oligomers from the HPLC and observing the stepwise formation of fragments in the presence of the phosphodiesterase. Thus, for example, the pentamer of p$\tilde{G}$p was isolated from the RPC-5 column (0.154 ODU in 4.5 ml of eluate), adjusted to pH 9.0 with HCl (1M), and 0.4 units of phosphodiesterase was added in 2 μl Tris-HCl (0.2M, pH 9.0, and 0.04M in $MgCl_2$). Periodic analysis demonstrated the sequential production of the peaks that were expected for tetramer, trimer, dimer, and monomer.

5.3 Results and Discussion

In the absence of a complementary polynucleotide template, no oligomers longer than trimer or tetramer were detectable from oligomerization of either of the acyclic analoques Imp$\tilde{G}$pIm or Imp$\tilde{A}$pIm. The major reaction was formation of the cyclic

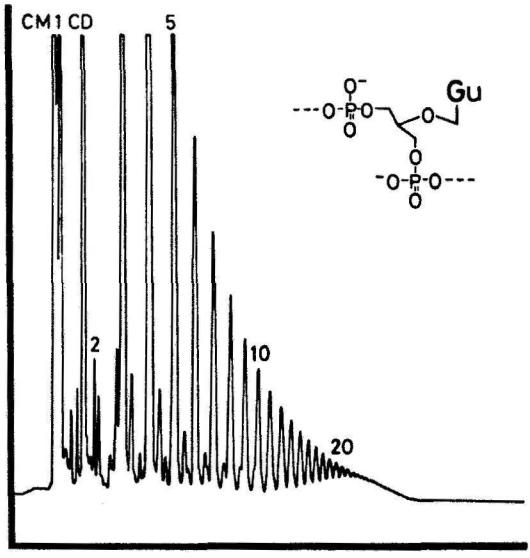
Table 5.1: Product distributions in the oligomerizations of ImpÃpIm, ImpG̃pIm, and ImpG̃pIm*

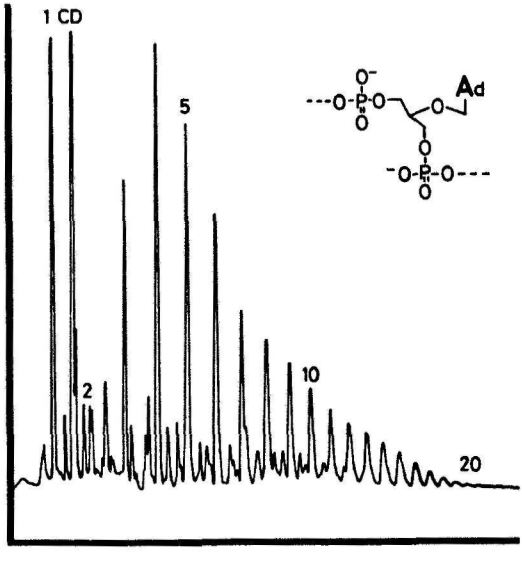
Monomer	Template	Unreacted monomer (%)	Cyclic monomer (%)	Incorporation of monomer into oligomers of length n (%)		
				$n \geq 2$	$n \geq 4$	$n \geq 10$
ImpÃpIm	-	4	86	5	-	-
ImpÃpIm	Poly(U)	3	58	36	30	7
ImpG̃pIm	-	4	89	7	-	-
ImpG̃pIm	Poly(C)	3	58	37	24	6
ImpG̃pIm	-	9	54	36	4	-
ImpG̃pIm	Poly(C)	7	54	38	9	0.3

* For reaction conditions see Experimental.

pyrophosphate of the monomer (Table 5.1). When template is present, a rapid template-directed oligomerization ensues, producing oligomers with chain lengths of 20 or above (Fig. 5.2^A and 5.2^B). These results confirm and extend those previously reported by Schwartz and Orgel (1985). The overall conversion of monomer to oligomers (37% after 1 week) is limited by the competing cyclization (Table 5.1). In contrast, ImpG̃pIm, which cyclizes to form a 10-membered rather than an 8-membered ring, produced more oligomerization in the absence of a template than the other acyclic analogues studied. However, the effect of a complementary template was greatly reduced (Table 5.1 and Figure 5.2^C). It is possible that the extended length of the backbone of this analogue produces a "strained" hetero-complex with the poly(C) template. We have not sought to optimize this reaction, but have used conditions which are optimal for ImpÃpIm and ImpG̃pIm so as to permit comparison.

The present investigation differs from our previous publications in that we have used an activating mixture of EDAC and imidazole, rather than presynthesized phosphoimidazolides. This procedure was adopted to minimize the formation of cyclic pyrophosphate during preparation of reaction mixtures. In spite of this modification in procedure, it is instructive to compare these results with those previously


CM 1 CD
5
2
10
20
Gu


1 CD
5
2
10
20
Ad

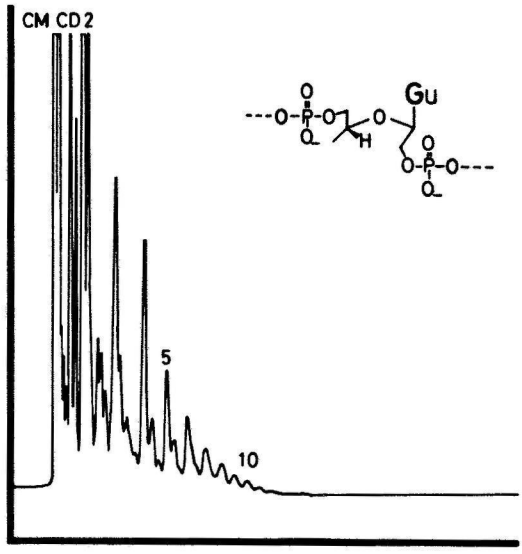


Figure 5.2: HPLC separations of the products of template-directed oligomerizations of analogues. **A** ImpÃpIm; **B** ImpG̃pIm; **C** ImpḠpIm. The numbers given the chain lengths of product oligomers. CM indicates the cyclic pyrophosphate of the monomer, and CD the cyclic pyrophosphate of the dimer. (The cyclic monomer of pÃp elutes earlier than the guanine-containing products and is not shown.)

obtained with the bisphosphoimidazolides of 2'- and 3'-deoxynucleosides (Fig. 5.1). In the 2'-deoxyribose series (ImpdGpIm and ImpdApIm), cyclization is relatively less important (Schwartz et al., 1987). Both ImpdGpIm and ImpdApIm react to form long oligomers in the absence of template, although at a much lower rate than the template-directed reaction. Presumably this is a consequence of the relatively rigid ribofuranose ring providing a less favorable geometry for cyclization of the monomer.

The oligomerization of the 3'-deoxynucleoside analogue Impd(3')ApIm was found to proceed very rapidly, even in the absence of a template, to produce high yields of oligomers. This high rate of oligomerization is probably due to the fact that internal cyclization for this monomer is almost totally prohibited. When poly(U) was added, only a modest increase in yields of the longest oligomers was observed (Visscher and Schwartz, 1988). It was suggested that the nine-atom structural unit of the product oligomers produced a less stable triple-helical complex with poly(U). Recent results, however, indicate that at lower monomer concentrations, where template-free oligomerization of the monomer is disfavored, substantial template catalysis occurs (J. Visscher, unpublished data). Clearly it will be of interest to extend our investigations of these reactions to a more selective range of conditions.

The avidity with which 1,3-bisphosphoimidazolides of glycerol cyclize creates a problem if these particular compounds are to be regarded as candidates for the hypothetical prochiral precursors of nucleic acids (Joyce et al., 1987). Related compounds which are sterically hindered may prove to have more favorable properties. Alternatively, a mechanism may be found to hydrolytically open or activate the cyclic compound so as to regenerate monomer.

References

Joyce, G.F., Schwartz, A.W., Miller, S.L., and Orgel, L.E. (1987) The case for an ancestral genetic system involving simple analogues of the nucleotides. Proc. Natl. Acad. Sci. USA 84, 4398-4402.

Joyce, G.F., Visser, G.M., van Boeckel, A.A., van Boom, J.H., Orgel, L.E., and van Westrenen, J. (1984) Chiral selection in poly(C)-directed

synthesis of oligo(G). Nature 310, 602-604.

McGee, D.P.C., and Martin, J.C. (1986) Acyclic nucleoside analogues: methods for the preparation of 2',3'-secoguanosine, 5'-deoxy-2',3'-secoguanosine, and (R,S)-9-[1-(2-hydroxyethyl)-2-hydroxyethyl]guanine. Can. J. Chem. 64, 1885-1889.

Ogilvie, K.K., Cheriyan, U.O., Radatus, B.K., Smith, K.O., Galloway, K.S., and Kennell, W.L. (1982) Biological active acyclonucleoside analogues. II The synthesis of 9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine (BIOLF-62). Can. J. Chem. 60, 3005-3010.

Schwartz, A.W., Visscher, J. Bakker, C.G., and Niessen, J. (1987) Nucleic acid-like structures II. Polynucleotide analogues as possible Primitive Precursors of nucleic acids. Origins of Life 17, 351-357.

Schwartz, A.W., and Orgel, L.E. (1985) Template-directed synthesis of novel, nucleic acid-like structures. Science 228, 585-587.

Visscher, J., and Schwartz, A.W. (1988) Nucleic acid-like structures III. Oligomerization of 3'-deoxyadenosine-2',5'-bisphosphate. J. Mol. Evol. 26, 291-293.

Zielinski, W.S., and Orgel, L.E. (1985) Oligomerization of activated derivatives of 3'-amino-3'-deoxyguanosine on poly(C) and poly(dC) templates. Nucleic Acids Res. 13, 2469-2484.

Zielinski, W.S., and Orgel, L.E. (1987) Oligoaminonucleoside phosphoramidates. Oligomerization of dimers of 3'-amino-3'-deoxynucleotides (GC and CG) in aqueous solution. Nucleic Acids Res. 15, 1699-1715.

Chapter 6

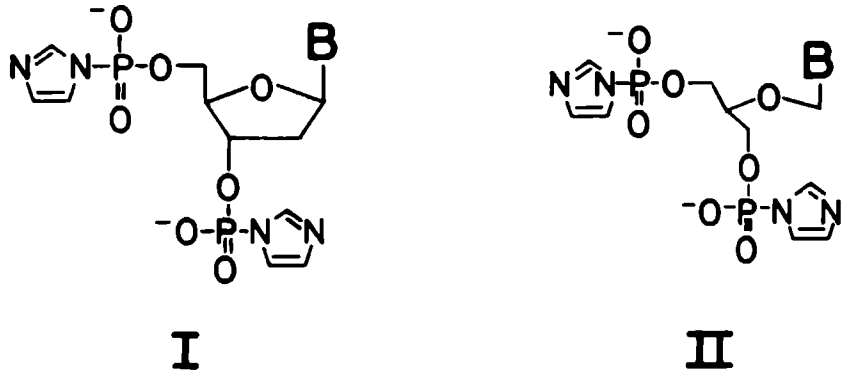
Mangenese-Catalyzed Oligomerizations of Nucleotide Analogs

Abstract

The synthesis of pyrophosphate-linked oligomers from the bisphosphoimidazolides of deoxyadenosine and deoxyguanosine, as well as from acyclic analogs of these nucleosides, is catalyzed much more effectively by Mn(II) than by Mg(II). The presence of Mn(II) reduces the extent of cyclization of the monomer-bisphosphoimidazolide and thereby increases the yields of oligomeric products. The Mn(II)-catalyzed oligomerization is less sensitive to the presence of a complementary polynucleotide template than is the Mg(II)-catalyzed reaction.

6.1 Introduction

In previous work (Schwartz et al., 1987; Visscher and Schwartz, 1988), we have described template-directed oligomerizations of 2'-deoxynucleoside bisphosphoimidazolides (Fig. 6.1, I), as well as of prochiral, acyclic analogs of these monomers (Fig. 6.1, II). Although the deoxynucleoside bisphosphoimidazolides also oligomerize more



I II

Figure 6.1: The structures of bisphosphoimidazolides of 2'-deoxynucleosides (**I**) and of acyclic nucleoside analogs (**II**). B = adenine or guanine.

slowly in the absence of a template, the acyclic analogs have only been oligomerized in the presence of a complementary polyribonucleotide template. The most serious limitation in both types of oligomerization is internal cyclization of the monomer. This process is so rapid in the case of the more flexible, acyclic monomers that significant oligomerization does not occur without a template. A similar problem due to monomer cyclization has also been encountered in another system (Hill et al., 1988). Internal cyclization is greatly reduced in the case of a sterically hindered monomer such as 3'-deoxyadenosine 2',5'-bis-phosphate (Visscher and Schwartz, 1987). Any interaction that would reduce the freedom of movement of one or both phosphate groups might also decrease the extent of monomer cyclization. Because it is known that Mn(II) interacts with the 5'-phosphate of a mononucleotide as well as with the base (Pezzano and Podo, 1980), we have compared the oligomerizations of a number

of monomers in the presence of Mn(II) with the more familiar reactions carried out with Mg(II).

6.2 Experimental

Enzymes and polynucleotides were purchased from Sigma Chemical Company. Bisphosphoimidazolides of dA and dG were synthesized as described in Schwartz et al. (1987). Reaction mixtures where prepared as described in Schwartz and Orgel (1985). These mixtures contained 0.1M ImpdApIm or ImpdGpIm, 0.2M $MgCl_2$ or $MnCl_2$, 0.1M NaCl, 0.5M Bis-Tris-HCl (pH 6.5), and, when added, 0.2M (monomer-equivalent) of the complementary polyribonucleotide template.

The synthesis of 9-[(1,3-dihydro-2-propoxy)methyl]guanine and 9-[(1,3-dihydro-2-propoxy)methyl]adenine, and the 3',5'-bisphosphorylation of these compounds has been described previously (Visscher and Schwartz, 1988). Reaction mixtures containing the bisphosphoimidazolides of $\tilde{A}$ and $\tilde{G}$ were prepared by in situ bis-imidazolation of $p\tilde{A}p$ and $p\tilde{G}p$ as described in Visscher and Schwartz (1988). The oligomerization mixtures contained 0.05M $Imp\tilde{G}pIm$ or $Imp\tilde{A}pIm$, 0.1M $MgCl_2$ or $MnCl_2$, 0.1M NaCl, and, when present, 0.1M (monomer-equivalent) of the complementary polyribonucleotide template in a total volume of 10 μl (0.4M imidazole as buffer, pH 6.5).

All reactions were carried out for 2 weeks at 0°C and were quenched by adding 2.5 μl KEDTA (1M, pH 9) and 7.5 μl $K_4P_2O_7$ (1M, pH 9). Storage was at -25°C. Before analysis, any surviving imidazolides and template, if present, were destroyed as described by Schwartz et al. (1987). Analysis was by HPLC on RPC-5 in 0.02M NaOH with a linear gradient of $NaClO_4$ (0-0.4M over 60 min) at a flow rate of 1.0 ml/min. Peak detection was by monitoring absorbance at 254 nm. Product yields given in Tables 6.1 and 6.2 were calculated from the HPLC elution profile by dividing the area under the peak(s) of interest by the sum of the area under all peaks in the profile.

Table 6.1: Product distributions in the oligomerization reactions of ImpdApIm and ImpdGpIm*

Monomer	Template	M^{2+}	Cyclic monomer (%)	Incorporation of monomer into oligomers of length n (%)		
				$n \geq 2$	$n \geq 4$	$n \geq 10$
ImpdApIm	-	Mg	44	35	9	-
ImpdApIm	-	Mn	26	59	32	6
ImpdApIm	Poly(U)	Mg	42	51	46	29
ImpdApIm	Poly(U)	Mn	30	63	33	4
ImpdGpIm	-	Mg	53	34	13	4
ImpdGpIm	-	Mn	35	56	30	7
ImpdGpIm	Poly(C)	Mg	39	55	46	27
ImpdGpIm	Poly(C)	Mn	33	62	38	14

* The data were collected as described in Experimental

6.3 Results and Discussion

The results are summarized in Tables 6.1 and 6.2. The substitution of Mn(II) for Mg(II) had a substantial effect on all oligomerizations in the absence of template. In the oligomerization of ImpdApIm, for example, the total yield of oligomers increased from 35% to 59%, with an even more dramatic increase from 9% to 32% in the yields of tetramer and longer oligomers (Table 6.1). These results can be understood if the interaction of Mn(II) with the phosphate groups as well as with the purine ring system reduces the freedom of motion of the phosphates and, consequently, the extent of internal cyclization of the monomer. The diminished effectiveness of the template in the presence of Mn(II) may be a consequence of destabilization of the complex, resulting from reduced base-stacking (Shin, 1973).

Although the total yields of oligomers from the acyclic monomers remain relatively low in the absence of template (Table 6.2), there is a significant improvement upon addition of Mn(II). This is especially noticeable in the case of ImpGpIm, where the oligomeric yield increased from 4% to 23%. Furthermore, it is only in the presence of Mn(II) that oligomers as long as the decamer were obtained from these acyclic

Table 6.2: Product distributions in the oligomerization reactions of ImpÃpIm and ImpG̃pIm*

Monomer	Template	M^{2+}	Cyclic monomer (%)	Incorporation of monomer into oligomers of length n (%)		
				$n \geq 2$	$n \geq 4$	$n \geq 10$
ImpÃpIm	-	Mg	76	6	-	-
ImpÃpIm	-	Mn	55	16	4	-
ImpÃpIm	Poly(U)	Mg	58	36	30	7
ImpÃpIm	Poly(U)	Mn	51	43	21	-
ImpG̃pIm	-	Mg	83	4	-	-
ImpG̃pIm	-	Mn	59	23	7	-
ImpG̃pIm	Poly(C)	Mg	58	37	24	6
ImpG̃pIm	Poly(C)	Mn	52	39	15	-

* The data were collected as described in Experimental

analogs in the absence of template (Fig. 6.2). Mn(II) seems to be quite specific in its interactions with the deoxynucleoside and analog bis-phosphates we have studied, as no other divalent metals were found to produce comparable results (Fe, Co, Ni, Cu, Zn, Pb and Cd were also tested). Highly specific effects of metals are known for template-directed oligomerizations of ribonucleoside-5'-phosphorimidazolides, both in the presence of a template (Bridson et al., 1981), and in the absence of template (Sawai, 1988). However, the mechanism responsible for these effects may be rather different, because the reactions involve the 2',3'-vicinal hydroxyl groups of ribose as nucleophiles, rather than a second phosphate group.

Acyclic nucleotide analogs have been suggested as possible prebiotic precursors of ribo- and deoxyribonucleotides (Joyce et al., 1987). It has been recognized, however, that the rapid internal cyclization of such monomers would constitute a serious obstacle in any proposed scheme of prebiotic synthesis (Visscher and Schwartz, 1987; Hill et al., 1988). The present results, although they by no means solve the problem, do demonstrate that mechanisms exist that would reduce cyclization under certain conditions. An ideal catalyst would have to combine the ability of Mn(II) to reduce intramolecular interactions between phosphate groups, with the properties of

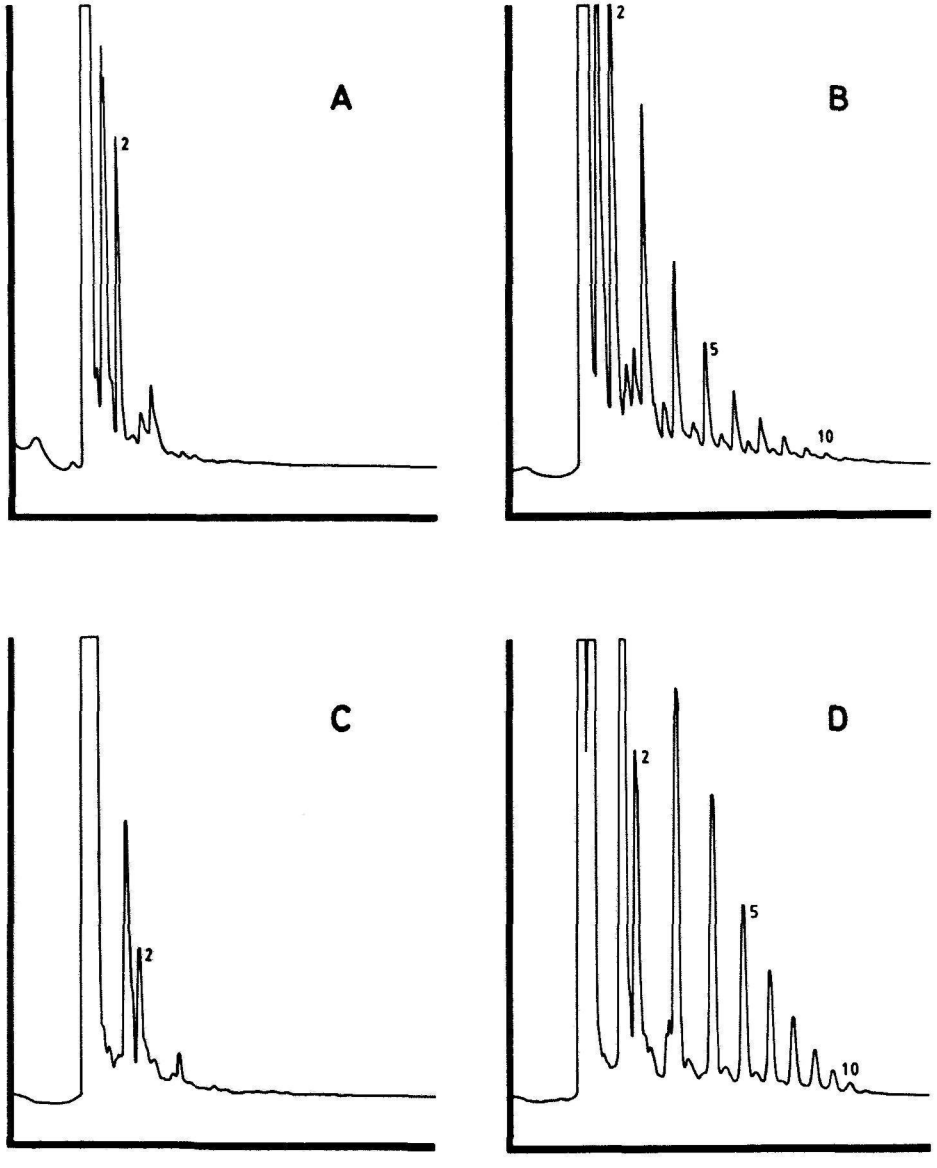


Figure 6.2: HPLC separation of the products of the oligomerizations of ImpÃpIm and ImpG̃pIm in the presence of Mg(II) or Mn(II). **A** ImpÃpIm + Mg(II); **B** ImpÃpIm + Mn(II); **C** ImpG̃pIm + Mg(II); **D** ImpG̃pIm + Mn(II). For conditions see Experimental.

Mg(II) in stabilizing duplex formation between monomers and template. Alternatively, other acyclic monomers may be found that are less subject to cyclization. It is premature to propose a specific mechanism or a specific monomer as a primitive precursor of nucleotides. Our goal is rather to test the general hypothesis that a "nucleic acid-like" system may exist that, while fulfilling the information storage requirements of such a precursor, is not subject to the synthetic and mechanistic difficulties involved in the contemporary ribose-based system.

References

Bridson, P.K., Fakhari, H., Lohrmann, R., Orgel, L.E., van Roode, M. (1981) Template-directed synthesis of oligoguanylic acids - metal ion catalysis. In: Origin of Life (Y. Wolman, Ed.). Reidel, Jerusalem, pp. 233-239.

Hill, A.R., Nord, L.D., Orgel, L.E., Robins, R.K. (1988) Cyclization of nucleotide analogues as an obstacle to polymerization. J. Mol. Evol. 28, 170-171.

Joyce, G.F., Schwartz, A.W., Miller, S.L., Orgel, L.E. (1987) The case for an ancestral genetic system involving simple analogs of the nucleotides. Proc. Natl. Acad. Sci. USA 84, 4398-4402.

Pezzano, H., Podo, F. (1980) Structure of binary complexes of mono- and polynucleotides with metal ions of the first transition group. Chem. Rev. 80, 365-401.

Sawai, H. (1988) Oligonucleotide formation catalyzed by divalent metal ions. The uniqueness of the ribosyl system. J. Mol. Evol. 27, 181-186.

Schwartz, A.W., Orgel, L.E. (1985) Template-directed synthesis of novel, nucleic acid-like structures. Science 228, 585-587.

Schwartz, A.W., Visscher, J., Bakker, C.G., Niessen, J. (1987) Nucleic acid-like structures II. polynucleotide analogues as possible primitive precursors of nucleic acids. Origins of Life 17, 351-357.

Shin, Y. (1973) Interaction of metal ions with polynucleotides and related compounds. XXII. Effect of divalent metal ions on the conformational

changes of polyribonucleotides Biopolymers 12, 2459-2475

Visscher, J., Schwartz, A.W. (1987) Nucleic acid-like structures III. Oligomerization of 3'-deoxyadenosine-2',5'-bisphosphate. J. Mol. Evol. 26, 291-293.

Visscher, J., Schwartz, A.W. (1988) Template-directed synthesis of acyclic oligonucleotide analogues. J. Mol. Evol. 28, 3-6.

Visscher, J., Bakker, C.G., van der Woerd, R., Schwartz, A.W. (1989) Template-directed oligomerization catalyzed by a polynucleotide analog. Science 244, 329-331.

Chapter 7

Oligomerization of Cytosine-Containing Nucleotide Analogs in Aqueous Solution

Abstract

Bisphosphoimidazolides of 2'-deoxycytidine and of its acyclic analog $\tilde{C}$ can be oligomerized in aqueous solution in the presence of Mn(II). Under certain conditions, a range of products extending to at least the 20mer can be obtained. These products are of interest as possible templates for oligomerization of the complementary monomers.

7.1 Introduction

Polyribonucleotides have been prepared in aqueous solution by using enzymatically synthesized polynucleotides as templates for the oligomerization of nucleoside phosphoimidazolides such as 2-MeImpG (Joyce et al., 1984). Pyrophosphate-linked polynucleotide analogs have similarly been synthesized by the oligomerization of bisphosphoimidazolides of 2'-deoxyribonucleosides or of acyclic nucleoside analogs on polynucleotide templates (Schwartz and Orgel, 1985; Visscher and Schwartz, 1988). Recently, a pyrophosphate-linked analog, oligo(pdCp), has been chemically synthesized and shown to catalyze the oligomerization of the complementary monomer (Visscher, Bakker et al., 1989). These studies have been undertaken as part of a program to develop models for primitive self-replicating systems. An obvious question to be answered is how the first templates for any such system might have arisen spontaneously. Pyrophosphate-linked polynucleotide analogs are also synthesized, albeit less efficiently, by oligomerization of bisphosphoimidazolides in the absence of a template (Schwartz et al., 1987). A limitation in these studies however, has been the observation that only the purine-containing analogs produce long oligomers in aqueous solution. Since oligomers containing largely purines are not capable of serving as templates in either of the systems which have been studied to date, the question arises as to how the first pyrimidine-rich templates might have formed. Sawai (1988) has reported that the oligomerization of ImpC in aqueous solution is catalyzed by Pb(II). Under these conditions, 2'-5' phosphodiester-linked oligomers are produced up to about the hexamer. It is not known whether 2'-5' linked oligonucleotides have template activity. In the pyrophosphate-linked system, we have found that manganese ions are more effective than magnesium in catalyzing the oligomerization of purine-containing analogs in aqueous solution (Visscher and Schwartz, 1989). We now report that the oligomerization of pyrimidine-containing analogs is also much improved in the presence of manganese and that under certain conditions, oligomers as long as the 20mer are formed.

7.2 Experimental

The preparation of 1-[(1,3-dihydroxy-2-propoxy)methyl]cytosine ($\tilde{C}$) was accomplished by condensation of silylated cytosine (Ogilvie et al., 1984) with 1,3-dibenzyloxy-2-chloromethoxypropane and a catalytic amount of tetra-n-butylammonium iodide in dry acetonitrile as described for the preparation of 1-[(1,3-dibenzyloxy-2-propoxy)methyl]guanine (Ogilvie et al., 1982). The protecting benzyl groups were removed by treating 1-[(1,3-dibenzyloxy-2-prooxy)methyl]cytosine with BCl_3 as described in Ogilvie et al. (1984).

$\tilde{C}$ was bisphosphorylated as described in Visscher and Schwartz (1988). The 3',5'-bisphosphate of 2'-deoxycytidine was prepared as described for the preparation of 2'-deoxyadenosine-3',5'-bisphosphate (Schwartz et al., 1987). pdTp was a gift of C.G. Bakker.

The bisphosphoimidazolide of dT was prepared as described in Schwartz et al. (1987). The bisphosphoimidazolides of dC or $\tilde{C}$ were prepared using a modification of the procedure described for the preparation of 5'-phosphoimidazolides of nucleotides by Joyce et al., (1984). The bisphosphates (70 μmol) of $\tilde{C}$ or dC were suspended in 1 ml of tetrahydrofuran plus 0.5 ml of triethylamine. To this suspension was added a solution containing 250 μmol of imidazole, 250 μmol of triphenylphosphine, 250 μmol of 2',2'-dithiodipyridine and 500 μl of triethylamine in 2 ml of tetrahydrofuran. After 24 h at room temperature the product was precipitated by treating with 100 μl of $NaClO_4$-saturated acetone and 100 ml acetone - diethylether (1:4). After washing the suspension 5 times with acetone - diethylether (1:4), the product was stored under vacuum over P_2O_5 and KOH.

Oligomerizations were carried out as described in Schwartz and Orgel (1985). The conditions are given in the tables.

After a reaction period of 2 or 4 weeks at 0°C the reaction mixtures were quenched by adding 9 μl of EDTA (1.0M, pH 9) and water to a total volume of 50 μl. Storage was at -25°C. Prior to analysis any surviving imidazolides were hydrolyzed overnight at room temperature by adding 2 to 4 μl of the quenched reaction mixture to 100 μl of sodium acetate (0.1M, pH 4.0).

Analyses were performed by HPLC on RPC-5 (Joyce et al., 1984) in 0.02M

NaOH with a linear gradient gradient of $NaClO_4$ (0-0.04M over 60 min) at a flow rate of 1.0 ml/min. Peak detection was by absorbance monitoring at 254 nm. The cyclic-monomers, cyclic-dimers and the pentamer of pdCp were verified by isolating these products from the RPC-5 column and treating with alkaline phosphatase and phosphodiesterase from venom, as described in Visscher and Schwartz (1988) and Schwartz et al. (1987).

7.3 Results and Discussion

The mechanism of oligomerization of bisphosphoimidazolides at pH 6.5 probably involves a gradual hydrolysis to produce free phosphate groups, which then attack a neighboring phosphorimidazolide, producing either an internucleotide or an internal pyrophosphate bond. We have previously reported that Mn(II) catalyzes the oligomerization of ImpdGpIm and ImpdApIm at pH 6.5 more effectively than Mg(II), probably due to an inhibition of internal cyclization of the monomer (Visscher and Schwartz, 1989). Table 7.1 compares the results for ImpdTpIm and ImpdCpIm at pH 6.5 with those previously obtained for ImpdApIm and ImpdGpIm. Although the total yield of oligomers produced from both pyrimidines is nearly doubled in the presence of Mn(II), there is no reduction in the extent of cyclization. This behavior can be correlated to some extent with reported differences in the structures of the Mn(II) complexes of purine and pyrimidine nucleotides. In the case of purine nucleotides, Mn(II) can interact with a phosphate group as well as with the purine ring system of the same molecule (Pezzano and Podo, 1980). This interaction probably has the effect of restricting the freedom of motion of one of the two phosphates, reducing the rate of the cyclization reaction and thereby favoring intermolecular condensation. A different situation exists with regard to the pyrimidines, for which Mn(II) can interact with the pyrimidine ring, but not simultaneously with a phosphate group of the same molecule (Pezzano and Podo, 1980). It is not clear, however, why the oligomerization yields are increased in the presence of Mn(II). Of possible relevance is the description of a 1:1 complex of Mn(II) with cytidine 5'-phosphate in the solid state (Aoki, 1976). In this structure phosphate groups of neighboring molecules are closely coordinated with each other to form a

Table 7.1: Oligomerizations at pH 6.5 (0.5M Bis-Tris HCl, 0.1M NaCl, two weeks at 0°C).

Monomer	MCl_2	CM	M	Incorporation of monomer into oligomers of length n (%)		
(0.1 M)	(0.1 M)	(%)	(%)	$n \geq 2$	$n \geq 4$	$n \geq 10$
ImpdTpIm	Mg	45	30	18	2	-
ImpdTpIm	Mn	50	12	34	3	-
ImpdCpIm	Mg	44	30	27	4	-
ImpdCpIm	Mn	45	7	49	12	trace
ImpdApIm	Mg	44	17	35	9	-
ImpdApIm	Mn	26	11	59	32	6
ImpdGpIm	Mg	53	14	34	13	4
ImpdGpIm	Mn	35	6	56	30	7

MCl_2 is the metal chloride, CM is the cyclic pyrophosphate of the monomer, and M is unreacted monomer.

three dimensional network. Conceivably, an interaction of this kind may provide a more favorable environment for internucleotide condensation than the Mg(II) complex.

It is the hydrolysis of one of the two phosphoimidazolide groups of each molecule which provides an opportunity for cyclization. We have therefore also studied the oligomerization of equimolar mixtures of the bisphosphoimidazolides and bisphosphates of dC and $\tilde{C}$ at pH 8.0, a pH at which the rate of hydrolysis is much reduced (Kanavarioti, 1986). These results are presented in Table 7.2 and Fig. 7.1 By increasing the concentrations to 0.2M, we were able to increase the total yield of oligomers for dC to 57%. The results with $\tilde{C}$ are less spectacular, although oligomers longer than the decamer can be detected in the chromatogram. The 13% yield of oligomers with chain lengths of 10 or more (23% of all oligomers) produced with dC is particularly interesting as these are potential templates for the oligomerization of ImpdGpIm. This fraction appears to extend beyond the 20mer (Fig. 7.1). The products undoubtedly contain a mixture of 3'-3', 3'-5' and 5'-5' linkages (Visscher, Woerd et al., 1989), and our expectation is therefore that their activity as templates

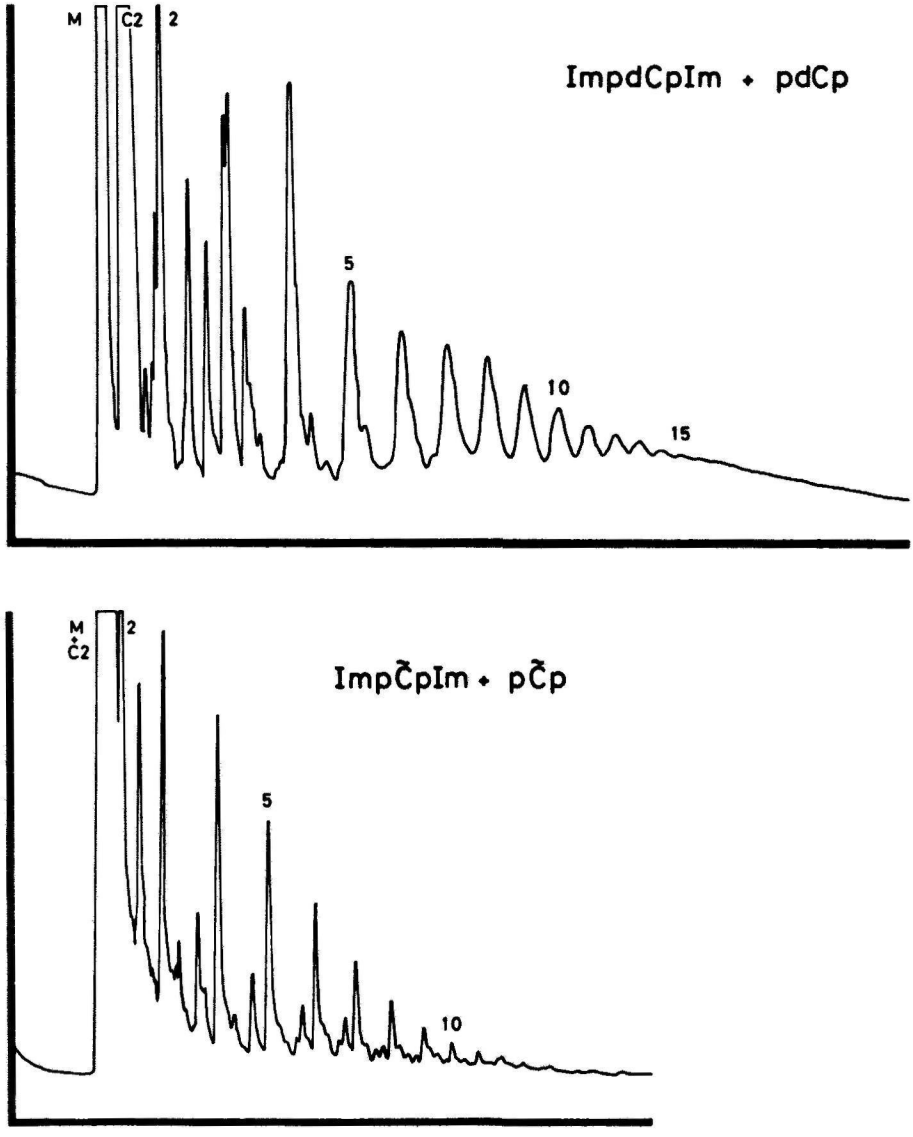


Figure 7.1: Products of the oligomerization of ImpdCpIm + pdCp and ImpC̃pIm + pC̃p at pH 8.0. (C2 is the cyclic dimer; cyclic monomer is not shown).

Table 7.2: Oligomerizations at pH 8.0 (0.5M Tris HCl, 0.1M NaCl, 4 weeks at 0°C).

					Incorporation of monomer into oligomers of length n (%)			
			CM	M				
ImpdCpIm	pdCp	MCl_2	(%)	(%)	$n \geq 2$	$n \geq 4$	$n \geq 10$	$n \geq 14$
0.05 M	0.05 M	Mg (0.2 M)	16	52	29	3	-	-
0.05 M	0.05 M	Mn (0.2 M)	14	47	38	21	1	-
0.10 M	0.10 M	Mn (0.6 M)	10	30	57	32	13	8
ImpC̃pIm	pC̃p							
0.10 M	0.10 M	Mn (0.6 M)	21	54	23	7	1	-

MCl_2 is the metal chloride, CM is the cyclic pyrophosphate of the monomer, and M is unreacted monomer.

will be less than that of the 3'-5' linked oligomers which we have recently reported on (Visscher, Bakker et al., 1989). A different situation may exist in the case of oligomers of the prochiral analog pC̃p (Fig. 7.1). These atactic oligomers possess a much more regular backbone geometry (Joyce et al., 1987) than the structurally heterogeneous oligomers of pdCp, and may therefore display template activity even when randomly oligomerized. We expect to be able to test this supposition shortly.

We do not suggest that structures of this type necessarily represent molecules which were abundant on the prebiotic Earth. No reasonable prebiotic synthesis has yet been demonstrated for either C̃ or dC. Although mechanisms which could account for bisphosphorylation are known (Lohrmann and Orgel, 1971; Schwartz et al., 1975), activation of the analog pC̃p is problematical because of the strong tendency to cyclize. Although this may be a formidable difficulty, it is in many ways less serious than the objections to β-D-ribonucleosides (Joyce et al., 1984; 1987). Furthermore, recent work on the oligomerization of cyclic pyrophosphates suggests that cyclization can be reversed (L.E. Orgel, personal communication). The studies described here may only be a first step toward developing a truly plausible model.

References

Aoki, K. (1976) X-Ray crystal structure of the 1:1 manganese-cytosine 5'-phosphate complex: Metal bonding to both O(2) of the base and phosphate. J.C.S. Chem. Comm. 748-749.

Joyce, G.F., Inoue, T., Orgel, L.S. (1984) Non-enzymatic template-directed synthesis on RNA random copolymers. Poly(C,U) templates. J. Mol. Biol. 176, 279-306.

Joyce, G.F., Schwartz, A.W., Miller, S.L., Orgel, L.E. (1987) The case for an ancestral genetic system involving simple analogues of the nucleotides. Proc. Natl. Acad. Sci. USA 84, 4398-4402.

Kanavarioti, A. (1986) Kinetics of the hydrolysis of guanosine 5'-phospho-2-methylimidazolide. Origins Life 17, 85-103.

Lohrmann, R., Orgel, L.E. (1971) Urea-inorganic phosphate mixtures as prebiotic phosphorylating agents. Science 171, 490-494.

Ogilvie, K.K., Cheriyan, U.O., Radatus, B.K., Smith, K.O., Galloway, K.S., Kennell, W.L. (1982) Biologically active acyclonucleoside analogues. II. The synthesis of 9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine (BIOLF-62). Can. J. Chem. 60, 3005-3010.

Ogilvie, K.K., Nguyen-Ba, N., Hamilton, R.G. (1984) A trihydroxy acyclonucleoside series. Can. J. Chem. 62, 1622-1627.

Pezzano, H., Podo, F. (1980) Structure of binary complexes of mono- and polynucleotides with metal ions of the first transition group. Chem. Rev. 80, 365-401.

Sawai, H. (1988) Oligonucleotide formation catalyzed by divalent metal ions. The uniqueness of the ribosyl system. J. Mol. Evol. 27, 181-186.

Schwartz, A.W., Orgel, L.E. (1985) Template-directed synthesis of novel, nucleic acid-like structures. Science 228, 585-587.

Schwartz, A.W., Visscher, J., Bakker, C.G., Niessen, J. (1987) Nucleic acid-like structures II. Polynucleotide analogues as possible primitive precursors of nucleic acids. Origins of Life 17, 351-357.

Schwartz, A.W., van der Veen, M., Bisseling, T., Chittenden, G.J.F. (1975) Prebiotic nucleotide synthesis - Demonstration of a geologically plausible pathway. Origins of Life 6, 163-168.

Visscher, J., Bakker, C.G., van der Woerd, R., Schwartz, A.W. (1989) Template-directed oligomerization catalyzed by a polynucleotide analog. Science 244, 329-331.

Visscher, J., Schwartz, A.W. (1989) Manganese-catalyzed oligomerizations of nucleotide analogues. J. Mol. Evol., in press.

Visscher, J., Schwartz, A.W. (1988) Template-directed synthesis of acyclic oligonucleotide analogues. J. Mol. Evol. 28, 3-6.

Visscher, J., Woerd, R. van der, Bakker, C.G., Schwartz, A.W. (1989) Oligomerization of deoxynucleoside-bisphosphate dimers. Origins Life 19, 3-6.

Chapter 8

Template-Directed Oligomerization Catalyzed by a Polynucleotide Analog

Abstract

A pyrophosphate-linked analog of polycytidylic acid has been synthesized and shown to catalyze the oligomerization of the complementary monomer 2'-deoxyguanosine 3',5'-bisphosphoimidazolide. Analogs of polynucleotides are of interest in studies of the origins of life as possible precursors of the first RNA molecules. These results demonstrate that such molecules are capable of serving as templates for further synthesis.

8.1 Introduction

Enzymatically synthesized polycytidylic acid [poly(C)] catalyzes the synthesis of oligoguanylic acids [oligo(G)'s] from an activated form of guanosine 5'-phosphate (Joyce et al., 1984). This system has been considered as a possible model for prebiotic replication of RNA. However, the inhibition of the reaction observed when both stereoisomers of the mononucleotide are present is not consistent with this role (Joyce et al., 1984). Poly(C) has also been shown to catalyze the oligomerization of both 2'-deoxyguanosine 3',5'-bisphosphoimidazolide (ImpdGpIm) and an acyclic analog of guanosine not based on ribose (Schwartz and Orgel, 1985; Schwartz et al., 1987; Visscher and Schwartz, 1988). In these latter template-directed reactions, the oligomers produced are linked by pyrophosphate, rather than phosphodiester linkages. Acyclic nucleic acid analogs with pyrophosphate backbones are possible precursors of the first RNA molecules (Joyce et al., 1987). It is important, therefore, that these molecules be capable of acting as templates for oligomerization. We have synthesized a pyrophosphate-linked polynucleotide analog based on 2'-deoxycytidine 3',5'-bisphosphate (pdCp) and now report that this product serves as a catalyst for template-directed oligomerization.

8.2 Experimental

Alkaline phosphatase (type III from *Escherichia coli*) was purchased from Sigma Chemical Company. Phosphordiesterase I from *Crotalus adamanteus* venom was purchased from P-L Biochemicals. DEAE-Sephadex and Q-Sepharose were purchased from Pharmacia. The monomer 2'-deoxyguanosine 3',5'-bisphosphoimidazolide was prepared as described (Schwartz et al., 1987). The protected deoxynucleoside N-4-diphenylacetyl-2'-deoxycytidine was prepared as described (Ti et al., 1982), with the modification that diphenylacetyl chloride was substituted for benzoyl chloride. The 5'-hydroxyl group was protected as the dimethoxytrityl derivative (Ti et al., 1982), and the 3'-hydroxyl was subsequently phosphorylated with the reagent bis[2-(methylsulfonyl)ethyl]phosphochloridate (Beld et al., 1984). The phosphorylation was performed as described (Woerd et

al., 1987), except that the solvent used was acetonitrile containing four equivalents of N-methylimidazole (Rooij et al., 1979). After removal of the protecting 5'-function in a mixture of $CF_3COOH/CHCl_3$ (1:25, for 15 s at 20°C), the phosphorothioate derivative was prepared as previously described (Woerd et al., 1987; Wreesmann et al., 1985). Removal of the protecting group from the 3'-phosphate was performed in a mixture of methanol and 25% NH_4OH (1:1, for 1 hour at 50°C). After evaporation of the solvent, the product was purified by chromatography on DEAE-Sephadex in a linear gradient of 0.05M to 0.4M triethylammonium bicarbonate (TEAB). The overall yield (based on deoxycytidine) after purification was about 30%. After evaporation of solvent (with ethanol to decompose TEAB, and repeated coevaporation with pyridine), a saturated solution of the dry product in pyridine was prepared (350 μmol monomer in pyridine to a total volume of approximately 0.5 ml). Oligomerization was achieved by addition of 3.5 mmol of dry, finely powdered iodine, which activates the phosphorothioate group by oxidation (Woerd et al. 1987; Wreesmann et al., 1985). The mixture was agitated in an ultrasonic bath for 1 hour and allowed to stand overnight. After hydrolysis of the DPA protecting groups in a mixture of CH_3OH and 25% NH_4OH (1:1, for 24 hours at 50°C), a preliminary purification was carried out on Q-Sepharose by eluting with 0.05M TEAB, to bring off nonnucleotide reaction products and cyclic pyrophosphate, followed by 2M TEAB. After evaporation of the solvent, the extent of oligomerization was determined by HPLC on RPC-5 (Joyce et al., 1984). The products were then separated on Q-Sepharose in a linear gradient of 0.05M to 0.6M TEAB (pH 7.5), which brought off products with chain lengths less than 4. Longer oligomers were recovered in 2M TEAB. This fraction was treated with alkaline phosphatase to remove terminal phosphate groups. For each optical density unit of oligomer, digestion was carried out with 0.1 unit of enzyme in 0.04M tris-HCl containing 0.02M $MgCl_2$ (pH 8), for 4 hours at 37°C. The oligomers were refractionated on Q-Sepharose in a linear gradient of 0.05M to 1.5M TEAB. All oligomers with chain lengths of 16 and longer were collected in one fraction to be used as templates. Oligomerizations were carried out as described in Schwartz and Orgel (1985). The conditions are given in Fig. 8.2. Before analyses, reactions were stopped and any surviving phosphorimidazolide groups were hydrolysed to phosphate as described in Schwartz et al. (1987). Analyses were performed

by HPLC on RPC-5 (Joyce et al., 1984) in 0.02M NaOH with a linear gradient of $NaClO_4$ (0-0.04M over 60 min) at a flow rate of 1.0 ml/min. Peak detection was by absorbance monitoring at 254 nm.

8.3 Results and Discussion

The monomer N-4-diphenylacetyl-2'-deoxycytidine 3'-O-phosphate, 5'-O-(S-4-methylphenyl)phosphorothioate (structure 1 in Fig. 8.1[A]) was synthesized and subjected to oligomerization. In the absence of other nucleophiles, reaction of the activated 5'-phosphate can only occur with a free 3'-phosphate group. After removal of the diphenylacetyl protecting groups, therefore, the major products expected were the cyclic pyrophosphate (structure 2 in Fig. 8.1[A], produced by intramolecular cyclization of the activated intermediate) and a series of 3',5'-pyrophosphate-linked oligomers of pdCp (structure 3 in Fig. 8.1[A]). The crude products were fractionated, and oligomers were subjected to alkaline phosphatase treatment to remove terminal phosphate groups.

All oligomers with chain-lengths of 16 and longer were collected in one fraction to be used as templates. We estimate that the lengths of oligomers in this fraction extended to about 40, with a mean of approximately 20. Analysis of the products by ^{31}P nuclear magnetic resonance (NMR) confirmed the absence of terminal phosphates and showed a resonance peak due only to internal pyrophosphate groups (the chemical shift relative to phosphoric acid was -9.5 ppm). Digestion with phosphodiesterase I from venom was used as previously described (Schwartz et al., 1987), together with high-performance liquid chromatography (HPLC) on RPC-5, to verify an isolated oligomer with a chain length of 8. As expected from previous studies (Schwartz and Orgel, 1985; Schwartz et al., 1987) on pyrophosphate-linked oligomers of 2'-deoxyguanosine 3',5'-bisphosphate (pdGp) and 2'-deoxyadenosine 3',5'-bisphosphate (pdAp), a series of oligomers of lengths 2 to 7 was produced, with ultimate conversion of all oligomers to monomer.

We compared the oligomerization of ImpdGpIm (Fig. 8.1[B]) in the absence of template with the oligomerization in the presence of oligo(pdCp) (Fig. 8.2). In the absence of a template, the primary reaction was cyclization of the monomer.

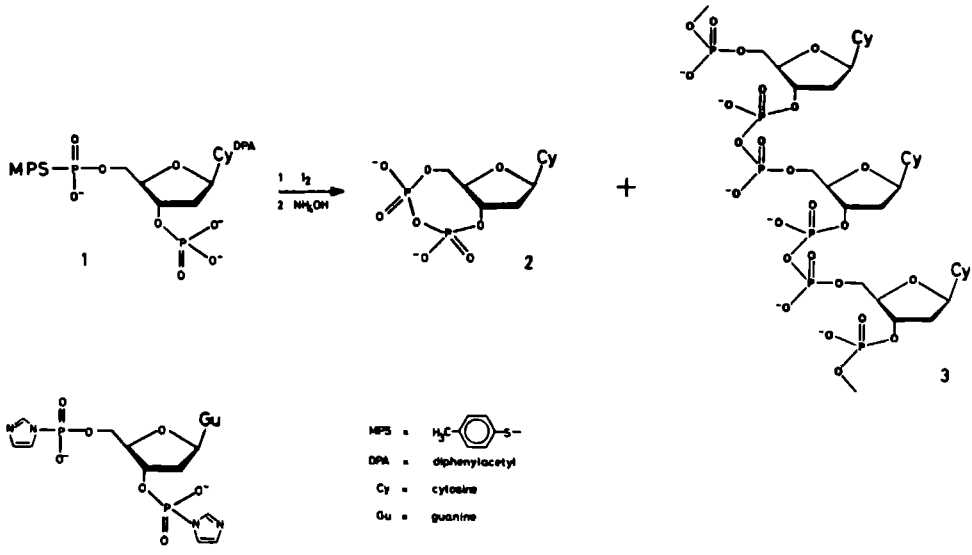


Figure 8.1: Structures of monomers and oligomers synthesized. (**A**) Oxidation of monomer (structure 1), followed by hydrolysis, produces cyclic pyrophosphate (structure 2) and oligomers of pdCp (structure 3). (**B**) The structure of the complementary, activated monomer ImpdGpIm.

Dimer (7%), cyclic dimer (6%), and trimer (2%) were the major oligomers formed. In the presence of oligo(pdCp), the total yield of oligomers increased from 17% to 36%. Much more significant, however, was the formation of product oligomers with chain lengths of at least 9 (longer products are obscured by the presence of the template). The control experiment established that the template is chemically stable under the conditions of the oligomerization. We verified that the products were pyrophosphate-linked by isolating the oligomer with a chain length of 6 from the RPC-5 column and following its sequential degradation by venom phosphodiesterase to yield pdGp. These results with a heterogeneous mixture of relatively short-chain oligo(pdCp) molecules are not dramatic when compared to oligomerization in the presence of enzymatically synthesized, high molecular weight poly(C) (Schwartz et al., 1987). However, they establish that the substitution of a pyrophosphate-linked backbone for the conventional one does not destroy the ability of the analog to

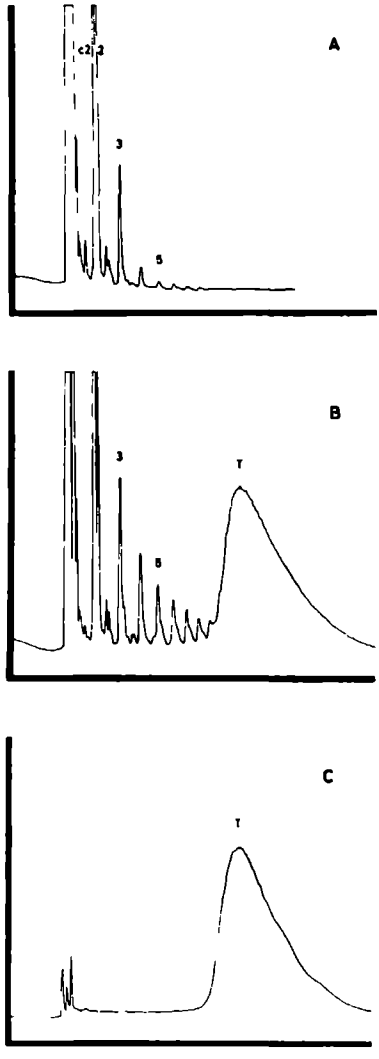


Figure 8.2: Oligomerization of ImpdGpIm. (**A**) A solution containing 0.025M ImpdGpIm, 0.2M $MgCl_2$, 0.1M NaCl, and 0.5M Bis-Tris HCl (pH 6.5) was incubated for 3 weeks at 0°. (**B**) Same as (**A**) but with 0.025M (monomer equivalent) oligo (pdCp). (**C**) Same as (B) but without ImpdGpIm. Oligomers with length 2, 3, and 5 are identified on the chromatograms; c2, cyclic dimer; and T, oligo(pdCp) template.

catalyze the synthesis of its complement.

These results have implications for theories on the origins of life. Arguments have been presented that purine and pyrimidine ribosides are highly implausible as prebiotic monomers (Joyce et al., 1987; Shapiro, 1988). The implausibility is related to the availability and properties of ribose rather than that of the purines and pyrimidines themselves. An acyclic, prochiral monomer based on glycerol bisphosphate and producing pyrophosphate-linked oligomers has been suggested as a possible evolutionary precursor to RNA (Visscher and Schwartz, 1988; Joyce et al., 1987). Although an actual demonstration of template-directed synthesis catalyzed by oligomers in which ribose or deoxyribose plays no part remains to be achieved, a step towards that goal has now been taken.

Referenties:

Beld, A., Claesen, C.A.A., Roersma, E.S., Schipper, W.J.M., Keiser, L.M., Tesser, G.I. (1984) Bis [2-(methylsulfonyl)ethyl] phosphorochloridate, a new phosphorylating agent. Recl. Trav. Chim. Pays-Bas 103, 196-202.

Joyce, G.F., Inoue, T., Orgel, L.S. (1984) Non-enzymatic template-directed synthesis on RNA random copolymers. Poly(C,U) templates. J. Mol. Biol. 176, 279-306.

Joyce, G.F., Schwartz, A.W., Miller, S.L., Orgel, L.E. (1987) The case for an ancestral genetic system involving simple analogues of the nucleotides. Proc. Natl. Acad. Sci. USA 84, 4398-4402.

Joyce, G.F., Visser, G.M., van Boeckel, A.A., van Boom, J.H., Orgel, L.E., Westrenen van, J. (1984) Chiral selection in poly(C)-directed synthesis of oligo(G). Nature 310, 602-604.

Rooij, J.F.M. de, Wille-Hazeleger, G., Deursen, P.H. van, Serjijn, J., Boom, J.H. van (1979) Synthesis of complementary DNA fragments via phosphotriester intermediates. Recl. Trav. Chim. Pays-Bas 98, 537-548.

Schwartz, A.W., Orgel, L.E. (1985) Template-directed synthesis of novel, nucleic acid-like structures. Science 228, 585-587.

Schwartz, A.W., Visscher, J., Bakker, C.G., Niessen, J. (1987) Nucleic

acid-like structures II. Polynucleotide analogues as possible Primitive Precursors of nucleic acids. Origins of Life 17, 351-357.

Shapiro, R. (1988) Prebiotic ribose synthesis: A critical analysis. Origins Life 18, 71-85.

Ti, G.S., Gaffney, B.L., Jones, R.A. (1982) Transient protection. Efficient one-flask synthesis of protected deoxynucleotides. J. Am. Chem. Soc. 104, 1316-1319.

Visscher, J., Schwartz, A.W. (1988) Template-directed synthesis of acyclic oligonucleotide analogues. J. Mol. Evol. 28, 3-6.

Woerd, R. van der, Bakker, C.G., Schwartz, A.W. (1987) Synthesis of P1,P2-dinucleotide pyrophosphates. Tet. Lett. 24, 2763-2766.

Wreesmann C.T.J., Fidder A., Veeneman G.H.. van der Marel G.A., van Boom J.H. (1985) S-4-methylphenyl-O.O-bis[1-benzotriazolyl]-phosphorothioate a versatile phophorylating agent. Tet. Lett. 26, 933-936.

Abbreviations

A	:	adenosine
Ã	:	9-[(1,3-dihydroxy-2-propoxy)methyl]adenine
C	:	cytidine
C̃	:	1-[(1,3-dihydroxy-2-propoxy)methyl]cytosine
G	:	guanosine
G̃	:	9-[(1,3-dihydroxy-2-propoxy)methyl]guanine
Ḡ	:	(R)-9-[1-(1-hydroxy-2-propoxy)-2-hydroxyethyl]-guanine
I	:	inosine
U	:	uridine
dA, dG, dI, and dC	:	2'-deoxynucleosides of adenine, guanine, hypoxanthine, and cytosine
dNp (N is A,G, or I)	:	the 3'-phosphate of dN
pdN (N is A,G, or I)	:	the 5'-phosphate of dN
pN (N is A,C,G, or U)	:	the 5'-phosphate of N
pN*p (N* is Ã,C̃,G̃, or Ḡ)	:	the bisphosphate of N*
Impd(3')ApIm	:	the 2',5'-bisphosphoimidazolide of 3'-deoxyadenosine
ImpdNpIm (N is A,C,G, or I)	:	the 3',5'-bisphosphoimidazolide of dN
ImpN*pIm (N* is Ã,G̃,C̃, or Ḡ)	:	the bisphosphoimidazolide of N*
2-MeImpG	:	the 2-methylimidazolide of G
Bis-Tris	:	bis(2-hydroxyethyl)imino-tris(hydroxymethyl)-methaan
DCC	:	dicyclohexylcarbodiimide
DMF	:	dimethylformamide
DMSO	:	dimethylsulfoxide

DPA	:	diphenylacetyl
EDAC	:	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
HPLC	:	high performance liquid chromatography
KEDTA	:	the potassium salt of ethylene diamine tetraacetic acid
NMR	:	nuclear magnetic resonance
MPS	:	methylphenylphosphorothioate
oligo(pdCp)	:	3'-5' pyrophosphate linked oligomers of pdCp
pdAppdAp or $(pdAp)_2$	:	P^1,P^2 3'-5' dinucleotide pyrophosphate of dA
poly(A)	:	polyadenylic acid
poly(C)	:	polycytidylic acid
poly(G)	:	polyguanylic acid
poly(U)	:	polyuridylic acid
TEAB	:	triethylammonium bicarbonate
Tris	:	tris(hydroxymethyl)aminomethane

Samenvatting

Een van de meest geciteerde hypothesen betreffende de chemische evolutie is de opvatting dat het leven zich heeft ontwikkeld vanuit RNA-moleculen die zichzelf zouden moeten reproduceren. De wijze waarop deze RNA-moleculen onder geologische condities zouden moeten worden gevormd, is echter onbekend. We stuiten hierbij op grote synthetische en mechanistische problemen. Om deze problemen te vermijden werd er gepostuleerd dat ons huidige genetische systeem geëvolueerd zou zijn uit een zichzelf reproducerend systeem waarin "primitieve" nucleotiden (achirale analogen) voorkomen. Dit onderzoek heeft een aanzet gegeven tot het bestuderen van een mogelijk kandidaat voor zo'n primitief systeem.

In hoofdstuk 2 worden de oligomerisatie reacties beschreven van bisfosforoimidazolaten van 2'-deoxyadenosine, 2'-deoxyguanosine en 2'-deoxyinosine. Onder optimale omstandigheden condenseren geactiveerde bisfosfaten van deoxyadenosine of deoxyguanosine tot gemengde oligomeren met 3'-3', 3'-5' en/of 5'-5' pyrofosfaatbruggen en met ketenlengten van circa 20 nucleosideresten. De oligomerisatie van geactiveerd deoxyinosine-bisfosfaat verloopt inefficiënt. Dit afwijkende gedrag werd verklaard door een unieke combinatie van stackings-interakties en hun onderlinge oriëntatie. Complementaire matrijzen kunnen de oligomerisatie reacties van bisfosfoimidazolaten afgeleid van 2'-deoxyadenosine en 2'-deoxyguanosine katalyseren. Hierbij treedt echter intramoleculaire cyclisatie op (vorming van 3'-5' cyclische pyrofosfaten) die door een complementaire matrijs niet geheel kan worden voorkomen.

In hoofdstuk 3 worden reacties beschreven van het bisfosfoimidazolaat van 3'-deoxyadenosine. De vorming van intramoleculaire pyrofosfaten binnen dit analogon wordt door sterisch minder goede bereikbaarheid belemmerd. Dit resulteert in een effeciënte oligomerisatie, waarbij 2'-2', 2'-5' en 5'-5' pyrofosfaatbindingen als "ruggegraat" ontstaan. Het complementaire polyuridylzuur katalyseert deze reactie.

In hoofdstuk 4 wordt het richtende effect van een polynucleïnezuur op de vorming van pyrofosfaatbruggen beschreven. Dit wordt gedemonstreerd met behulp van oligomerisatie reacties van dimeren van 3'-fosforo-2'-deoxyadenylzuur (pdAp) waarin dus pyrofosfaat bindingen tussen de 3'-3', 5'-5', en 3'-5' posities voorkomen. Van de drie types dimeren wordt de 3'-5' gekoppelde dimeer van pdAp het effectiefst gekata-

lyseerd door zijn complementaire matrijs. Hoewel de matrijs de oligomerisatie van de symmetrische pyrofosfaten (3'-3' en 5'-5') eveneens katalyseert, is de toename van juist de langere oligomeren zeer klein tot nihil.

In hoofdstuk 5 worden oligomerisatie reacties van bisfosforoimidazolaten van acyclische analogen van dA en dG beschreven. In deze analogen is de base via een methyleengroep met de secondaire hydroxylgroep van glycerol verbonden. Ze kunnen worden opgevat als mogelijke prebiotische voorlopers van nucleotiden. Vooral in deze processen wordt de intramoleculaire pyrofosfaatvorming als storende nevenreactie gevonden. Complementaire polyribonucleïnezuur matrijzen herkennen deze analogen en hebben een sterk katalytisch effect op de oligomerisaties.

In hoofdstuk 6 worden de oligomerisaties van de bisfosforoimidazolaten van 2'-deoxynucleosiden en hun acyclische analogen in tegenwoordigheid van mangaan beschreven. De vorming van intramoleculaire pyrofosfaten uit geactiveerde bisfosfaten, zoals vermeld in hoofdstukken 2 en 5, kan vertraagd worden door mangaanionen. Dit verschijnsel wordt verklaard door de vorming van complexen waarbij zowel de purinebase als de fosforzuur groep aan C-5' betrokken zijn, hetgeen de beweeglijkheid van deze fosfaatrest beperkt. Het nadeel van deze remming door mangaanionen is, dat tevens de katalyse door een polyribonucleïnezuur-matrijs ongunstig wordt beinvloed. Vermoedelijk is door de complexering van mangaan en de purinebasen de vorming van de Watson-Crick-baseparen voor de matrijs gekatalyseerde reacties niet meer optimaal.

In hoofdstuk 7 is onderzocht hoe de oligomerisatie verloopt van bisfosforoimidazolaten van de pyrimidine nucleosiden en hun achirale analogen. In dit geval blijkt mangaan de intramoleculaire pyrofosfaatvorming niet te beïnvloeden. De oligomerisatie wordt door mangaan echter wel gunstig beïnvloed. Onder optimale omstandigheden kunnen lange oligomeren van zowel deoxyribose-bevattende monomeren als van de acyclische analogen gesynthetiseerd worden.

In hoofdstuk 8 wordt ingegaan op een belangrijke eigenschap van een primitief genetisch systeem: het moet de synthese van zijn complementaire vorm kunnen katalyseren. Om dit te controleren werden aan de hand van oligomeren van 3'-fosforo-2'-deoxycytidylzuur oligomerisatie reacties bestudeerd van het complementaire derivaat 3'-fosforo-2'-deoxyguanylzuur. De katalytische activiteit van de

matrijs werd aangetoond.

Curriculum Vitae

Johannes Visscher werd geboren op 13 oktober 1956 te Appingedam. Na het doorlopen van het middelbaar onderwijs (Mavo-4 (behaald in 1973), Havo (behaald in 1975), en V.W.O. (behaald in 1977)) studeerde hij Scheikunde aan de Rijksuniversiteit Groningen. In 1980 behaalde hij het Kandidaatsexamen S1. Het Doctoraalexamen met als hoofdvak Organische Chemie, onder leiding van prof. dr. R.M. Kellogg, en als bijvak Biochemie werd in 1984 behaald. Vanaf februari 1985 was hij werkzaam als wetenschappelijk medewerker aan het Laboratorium voor Exobiologie van de Katholieke Universiteit te Nijmegen, waar hij onder leiding van prof. dr. A.W. Schwartz het in dit proefschrift beschreven onderzoek verrichtte.

STELLINGEN

I

Anders dan Zielinski en Orgel doen vermoeden geven de oligomerisatie reacties van 3'-glycylamido-3'-deoxynucleotiden geen verklaring voor het ontstaan van de eerste matrijzen.

W.S. Zielinski and L.E. Orgel,
J. Mol. Evol. 29, 367-369 (1989).

II

Het door retro-synthese herleiden van biologische moleculen naar een gering aantal uitgangsstoffen is, mits deze uitgangsstoffen prebiotisch verklaard kunnen worden, een waardevolle aanvulling op de "traditionele prebiotische chemie".

G. Ksander, G. Bold, R. Lattmann, C. Lehmann, T. Früh,
Y. Xiang, K. Inomata, H. Buser, J. Schreiber, E. Zasss
und A. Eschenmoser,
Helv. Chim. Acta 70, 1115-1172 (1987).

III

Enkele enzym-behandelingen rechtvaardigen niet de conclusie, dat fosfordiësterases (snake venom en spleen) geen 2'-nor-2'-deoxynucleotiden herkennen.

K.K. Ogilvie, N. Nguyen-Ba, M.F. Gillen,
B.K. Radatus, U.O. Cheriyan, and H.R. Hanna,
Can. J. Chem. 62, 241-252 (1984).

IV

De chromatografische analyses van de oligomerisatie reacties van de bisfosfoimidazolides van 9-[3-hydroxy-2-(hydroxymethyl)prop-1-yl]adenine wijzen op het gebruik van een mengsel van 9- en 7- adenine gekoppelde verbindingen.

M. Tohidi and L.E. Orgel,
J. Mol. Evol. 28, 367-373 (1989).

V

De identificatie van het gesynthetiseerde 2,3-cyclopyrofosfoglyceraat door middel van ^{31}P-NMR van het reactie mengsel is onvoldoende.

S. Kanodia and M.F. Roberts,
Proc. Natl. Acad. Sci. USA 80, 5217-5221 (1983)

VI

Het is voorbarig te veronderstellen, dat de ancestrale synthese van purine bevattende nucleotiden via het tussenprodukt formylglycinamidine ribonucleotide analoog aan de biosynthetische route verloopt.

G. Wächtershäuser,
Proc. Natl. Acad. Sci. USA 85, 1134-1135 (1988).

VII

De voorstelling, dat RNA is ontstaan vanuit de "prebiotische soep van Haldane en Oparin" komt overeen met "generatio spontanea".

J.E. Darnell and W.F. Doolittle,
Proc. Natl. Acad. Sci. USA 83, 1271-1275 (1986).

VIII

De invoering van enkele geldautomaten leidt tot verplaatsing van de drukte voor het loket naar buiten het gebouw.

IX

Toenemende automatische functies van een fototoestel geven geen garantie voor een technische en artistieke verbetering van de foto.

J. Visscher
Nijmegen, 8 januari 1990